GASTROINTESTINAL AND EPICUTANEOUS SENSITIZATION TO PEANUT

IMPACT OF GASTROINTESTINAL AND SKIN BARRIER DISRUPPTION ON SENSITIZATION TO PEANUT

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ABSTRACT

It has been suggested that environmental factors substantially contribute to the increased prevalence of peanut allergy in industrialized countries. Specifically, the role of disrupted barrier integrity in the gastrointestinal tract has been implicated in the development of food allergy. The use of non-steroidal anti-inflammatory drugs (NSAIDs), which increase intestinal permeability, for the treatment of pain and fever is prevalent in industrialized countries. Therefore, the first aim of this study was to determine whether treatment with indomethacin, a prototypical NSAID, would act in an adjuvant like manner to facilitate sensitization to co-administered peanut protein. Furthermore, we investigated whether indomethacin increases susceptibility to anaphylaxis following oral challenge with peanut in sensitized mice.

First, a short model of cholera toxin-mediated sensitization to peanut was developed. Mice were given 1 mg of peanut protein and 5 μg of cholera toxin by oral gavage for 10 consecutive days. This resulted in a robust anaphylactic response and increased peanut-specific IgG1, but not IgE, two weeks following treatment. Mice exposed to peanut during a 10-day treatment with indomethacin (5 mg/kg on alternating days or 3.5 mg/kg daily) did not develop peanut-specific immunoglobulins or anaphylaxis following systemic challenge with peanut protein. Furthermore, treatment with two 5 mg/kg doses of indomethacin 24 and 1 hour before oral challenge did not facilitate anaphylaxis in peanut-sensitized mice. Therefore, we concluded that NSAID treatment is unlikely to play a role in the increased prevalence of peanut allergy, and that NSAID treatment does not increase susceptibility to peanut-induced anaphylaxis in sensitized mice.

The second part of this study aimed to develop a short model of epicutaneous sensitization, and address the impact of epicutaneous exposure to peanut during infancy. We investigated the role of site of exposure, duration of exposure, epidermal integrity, strain and age in epicutaneous sensitization. 10 consecutive days of epicutaneous exposure to 20 μg of peanut protein through tape stripped skin induced robust anaphylaxis following i.p. challenge. Neither 7 days of exposure through tape stripped skin, nor 10 days of exposure through intact skin facilitated sensitization. The strength of sensitization was strain-dependent; peanut-specific IgG1 was increased equally in both C57BL/6, and BALB/c mice, and to a greater extent in C3H/HeJ mice. Peanut-specific IgE was increased in both BALB/c and C3H/HeJ mice three weeks following peanut exposure. Epicutaneous exposure beginning one day after birth did not facilitate the development of either peanut-specific IgE or IgG1 in BALB/c mice, or anaphylaxis following systemic challenge. Beginning exposure at 2 weeks of age resulted in peanut-specific IgE and IgG1 production, as well as a robust anaphylaxis following i.p. challenge. Therefore, the development of peanut allergy through epicutaneous exposure is age dependent, and exposure during the neonatal period results in a hyporesponsive response.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

CPE	Crude Peanut Extract
DC	Dendritic Cell
Ig	Immunoglobulin
IL-	Interleukin
i.p.	Intraperitoneal
i.v.	Intravenous
LC	Langerhans Cell
LN	Lymph Node
PA	Peanut Allergy
PN	Peanut
s.c.	Subcutaneous
TS	Tape Strip

CHAPTER 1: INTRODUCTION

STATE OF THE PROBLEM: PREVALENCE AND ETIOLOGY OF PEANUT ALLERGY

Allergy is defined as an aberrant immune response to a normally innocuous antigen. Allergic individuals are characterized by the presence of antigen-specific immunoglobulins, specifically IgE. Peanut allergy (PA) affects approximately 1.5% of the Canadian population, and unlike most other food allergies, which are outgrown by adulthood, persists throughout life in 80% of affected individuals [1, 2]. PA is often diagnosed following a reaction to the first known peanut exposure [3, 4]. Following ingestion of peanut, many allergic individuals undergo a reaction that can produce symptoms ranging from a cutaneous rash, some difficulty breathing and gastrointestinal upset, to a severe reaction, termed anaphylaxis, that may include life threatening cardiovascular collapse and death. Despite attempts of strict dietary avoidance, accidental ingestion of peanut-containing products occurs in approximately 14% of peanut allergic individuals annually [5]. Furthermore, food-induced anaphylaxis accounts for approximately 30-50% of all anaphylaxis-related hospitalizations, and 59% of food-anaphylaxis fatalities [6]. Systemic administration of epinephrine following accidental ingestion of peanut is the only approved and effective treatment. The prevalence of PA in Canada and other developed countries has been increasing over the past 20 years suggesting that, in addition to genetic factors, environmental factors may play a significant role in the development of PA [1, 7].

The mechanisms through which an individual becomes allergic to foods are largely unknown. Clinical studies have provided correlative evidence linking a number of environmental or lifestyle factors to the development of PA. Among these, disturbances of the barrier integrity in both the GI mucosa and skin have been of paramount interest. Patients with either Crohn's Disease or Ulcerative Colitis may have impaired development of oral tolerance to foods, leading researchers to hypothesize that chronic inflammation in the GI tract may also facilitate food allergy [8, 9]. Furthermore, it has been shown that patients with food allergy frequently have increased intestinal permeability [10]. However, these studies do not allow us to establish whether the relationship between PA and altered intestinal permeability is causative or simply correlative. As for the skin, recent studies have shown that children with peanut allergy were more frequently exposed to peanut oil-containing skin creams and were more likely to suffer from oozing, crusted rashes during infancy [11]. In addition, it has been recently reported that there is a strong correlation between a defect in the filaggrin gene (encodes filaggrin, a protein essential for the integrity of the epidermis) and peanut allergy. Filaggrin mutations remained a significant risk factor even after accounting for the presence of atopic dermatitis [12]. These studies provide intriguing hypotheses, but inherent limitations of human studies preclude discerning the precise relationship between these events.

CELLULAR MECHANISMS OF PEANUT-INDUCED ANAPHYLAXIS

Peanut allergy is classified as a type I immediate hypersensitivity reaction. Following sensitization to peanut, subsequent exposure initiates a systemic effector response, potentially including anaphylaxis, which is mediated through a number of immunological pathways. Peanut-specific IgE binds to the high affinity FcεRI on mast cells (MCs) or basophils in sensitized individuals. Following secondary exposure, peanut cross-links the FcεRI-bound IgE, resulting in degranulation of the target cells [13, 14]. During degranulation, an array of cytokines, chemokines and preformed mediators such as histamine, heparin, serotonin, and proteases are rapidly released. These mediators can directly or indirectly increase vascular permeability, leading to a decrease in blood pressure and increase in the cellular concentration of circulating blood cells (measured by hematocrit), constriction of smooth muscle, and inflammatory cell recruitment [15-17].

Recently, our laboratory showed the importance of additional, non-IgE-MC pathways in mediating PIA. Phagocytes (including macrophages) were shown to play a role in an IgG1-dependent pathway of anaphylaxis. Indeed, peanut-sensitized MC deficient mice exhibit a substantially reduced, but not completely eliminated anaphylactic response following peanut challenge that was entirely abrogated following phagocyte depletion. Furthermore, IgE-deficient mice remain susceptible to anaphylaxis (albeit significantly reduced); however, blocking IgG1 in these mice completely eliminates anaphylaxis [17]. Thus, several pathways are involved to varying degrees in the pathogenesis of anaphylaxis.

MECHANISMS OF SENSITIZATION

Sensitization, the focus of this study, includes a series of events that culminate in the production of antigen-specific immunoglobulins, which bind to mast cells, resulting in degranulation following secondary exposure to the antigen. Following antigen uptake by dendritic cells (DCs), signaling by microbial products or other molecules (cytokines) triggers upregulation of MHC II and costimulatory molecules that induce naïve $CD4^+$ T cells to differentiate into Th2 cells. The outcome of the DC-T cell interaction is thought to be determined by a combination of antigen specific factors, as well as the route and dose of administration [18]. Recent interest in epithelial-derived cytokines (TSLP, IL-25, IL-33, GM-CSF) has provided some important insights into how DCs are able to induce such a wide variety of T cell responses. However, recent data from our laboratory has demonstrated that IL-33, but not TSLP or IL-25 is required for the initiation of peanut allergy (manuscript submitted). Thus, the route and nature of antigen exposure may influence the type of cytokines secreted by epithelial cells, and condition DCs to promote Th2 cell differentiation. Upon interaction with $CD4^+$ T cells that secrete Th2 cytokines, B cells also mature and differentiate into plasma cells that produce antigen-specific immunoglobulins (IgE and IgG1) which bind to the high affinity FcεRI and FcγRIII on various cell types [19].

While animal models have provided a strong platform for the study of effector mechanisms of anaphylaxis, knowledge of the mechanisms of sensitization are largely based either on clinical correlations or animal models that employ the use of strong immunological adjuvants, such as the mucosal adjuvant cholera toxin, or systemic aluminum-based adjuvants, to initiate an allergic response {N. LYCKE, 2006 #113}. Thus, little is known concerning the specific role that lifestyle factors may play in the increasing prevalence of PA in North America.

Allergen exposure necessarily occurs at barrier surfaces (skin, lungs, GI tract). These barriers play integral roles in sustaining life by balancing essential functions (i.e. thermoregulation, absorption of essential nutrients) with protection from pathogens, as well as preventing overt inflammation to commensal organisms and following trauma or infection. To maintain such an exquisite balance, barrier surfaces are equipped with resident immune cells that are ideally located to initiate not only a rapid immune response, but also a regulatory response to prevent excess inflammation that would lead to tissue damage {Clark, #91}.

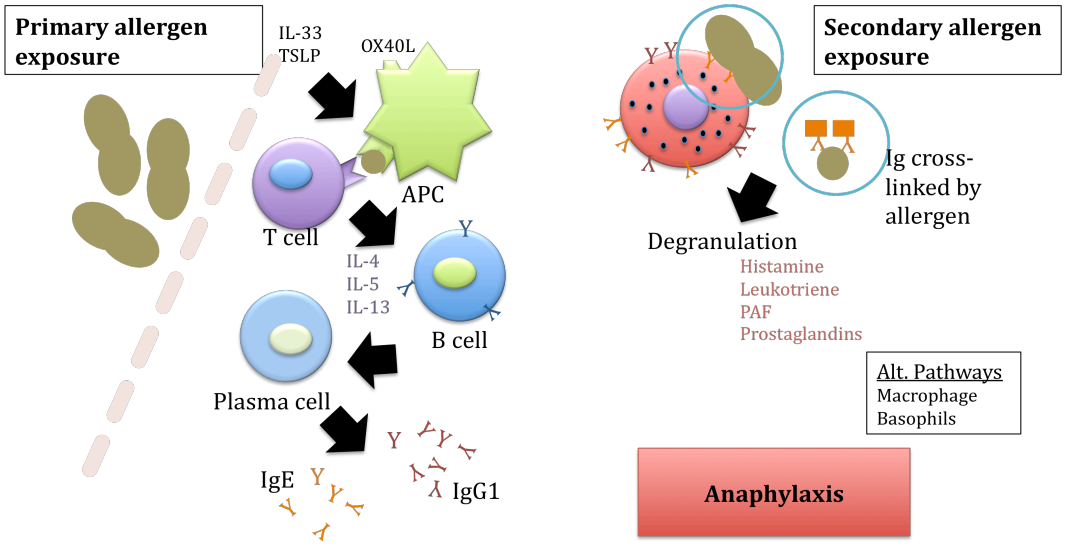

Figure 1: Mechanisms of Peanut Allergy. Sensitization involves interactions between structural or epithelial cells, epithelium-associated cytokines, T cells, APCs, B cells, and leads to the production of Th2 cytokines and antigen-specific IgE and IgG1 (left). Anaphylaxis may occur via a number of pathways, involving degranulation of mast cells, macrophages, or basophils following secondary allergen exposure in sensitized individuals (right).

ORAL SENSITIZATION

ORAL TOLERANCE

Sensitization to food allergens is thought to occur as a consequence of the breakdown or lack of induction of oral tolerance. Oral tolerance is the natural immunological response to ingested food antigens. Following oral exposure, antigens are actively taken up by enterocytes, M cells at Peyer's patches (lymphatic tissue), or by the dendrites of tolerogenic DCs that protrude into the intestinal lumen [20]. Under homeostatic conditions, epithelial cells in the intestine produce large amounts of the

regulatory molecules IL-10, TGF-β, retinoic acid, and prostaglandin E2. These molecules condition DCs, to induce IL-10- and/or TGF-β-producing regulatory T cells (Treg) (Tr1 and Th3), and also induce plasma cell production of neutralizing antigen-specific antibodies IgA and IgG4. Antigen-specific Tregs prevent the activation of effector T cell responses against food antigens [20, 21].

MECHANISMS OF ORAL SENSITIZATION

Although the route of sensitization to peanut has recently come into question, it was traditionally thought that the GI system, with the essential role of processing food antigens, was the most likely site. Peanut allergy is primarily diagnosed in young children following the first known exposure to peanut. Consequently, emphasis has been placed on the role of maternal consumption of peanut during pregnancy and lactation in sensitization of the newborn to peanut [22]. Indeed, peanut protein can be detected in breast milk from approximately 50% of lactating mothers 1-6 hours following ingestion [22]. However, studies have not provided sufficient evidence to link either consumption of peanut during pregnancy or during lactation to PA in children [23] [24]. Early introduction of peanut to children and the dose of peanut have also been implicated in the development of PA; however, studies have again not shown sufficient evidence to suggest a role for either timing or dose [25]. Sensitization to peanut through cross-reactivity with soy epitopes from soy formulas has been both supported and refuted in clinical studies [11]. Furthermore, children in China, who have comparable peanut intake to North American children, have a much lower rate of PA [26]. This has led to the suggestion that the method of processing (fried or boiled in China versus roasted in North America) impacts the allergenicity of peanut [26]. Indeed, it was found that boiled and fried peanuts have significantly less of the major peanut allergen Ara h 1, and the binding capacity of IgE to Ara h 2 and Ara h 3 was significantly lower than roasted peanut [26]. Additionally, it has been shown that Jewish children in Israel, where peanut is introduced earlier in life and in greater quantities, have a 10 fold lower incidence of peanut allergy than Jewish children in the United Kingdom [27]. Further environmental factors that have been suggested to contribute to the development of PA are infections and the use of antibiotics that may, together or separately, influence oral tolerance or sensitization by altering commensal flora and increasing intestinal permeability. The use of antacids that decrease acidity of the stomach, preventing full digestion of the resistant peanut proteins Ara h 1 and Ara h 2 has also been suggested as a possible mechanism of sensitization [28, 29].

PHYSIOLOGY OF THE GI SYSTEM

A single layer of epithelial cells separates the gastrointestinal lamina propria from the intestinal lumen [30]. Most food proteins are digested into small di- or tripeptides or amino acids by proteases in the stomach, and are further broken down by pancreatic and small intestinal proteases in the small intestine. Peptides may pass through the epithelium either transcellularly (through epithelial cells) or paracellularly (around epithelia cells). Large molecules, including proteins up to 1500 Daltons (Da), pass through the transcellular pathway, where they are endocytosed by epithelial cells and subject to

lysosomal degradation. Smaller peptides, less than 600 Da, are able to pass between the tight junctions, desmosomes, and adherens junctions that join epithelial cells, and peptides between 500-1500 Da may leak through these junctions in miniscule amounts [31].

The majority of protein constituents are absorbed in the small intestine. Within this tissue, in addition to epithelial cells, three separate immune compartments are also capable of antigen uptake: the gastrointestinal associated lymphatic tissue (GALT) which includes Peyer's patches, organized lymphoid follicles, and the appendix, intraepithelial lymphocytes (IEL), and lamina propria lymphocytes (LPL) [20]. M cells, present over Peyer's patches and isolated lymphoid follicles, uptake soluble and aggregated proteins, which are then taken up by resident DCs and, under normal conditions, lead to induction of tolerance [20]. Peyer's patches and the GI draining mesenteric lymph nodes are the sites of primary immune responses and induction of tolerance [20]. Following high doses of ingested protein, T cell anergy and apoptosis are observed and, following low doses of ingested protein, tolerogenic Tregs are produced in both the Peyer's patches and mesenteric lymph nodes [20]. Further studies have demonstrated that although Peyer's patches are not necessary for the induction of oral tolerance, MLN are both necessary and sufficient. Thus, the amount of protein that reaches the LB (and hence intestinal permeability) can contribute to the immunological response initiated [32].

IMMUNOLOGY OF THE GI SYSTEM

Epithelial cells and resident immune cells in the gut are the initial site of contact for orally ingested food allergens. The GI system must balance protection from pathogens and prevention of immunopathology. It is therefore beneficial to remain tolerant to food antigens, even in the presence of minor inflammation. To accomplish this mission, the GI tract maintains one of the most highly regulatory environments in the body.

DCs in the intestine have been shown to have an intrinsically greater ability to induce $FoxP3^+$ regulatory T cells than splenic DCs as a result of their ability to convert latent TGF-β into its active form. They may also induce Tregs through IL-10, retinoic acid, IL-27, vitamin D, or indoleamine-2,3-dioxygenase mediated pathways [33]. Furthermore, these cells have an impaired capacity to induce Th17 responses, and a marked decrease in the expression of the co-stimulatory molecules CD80 and CD86, which can only be overcome in the presence of strong inflammatory signals such as IL-1 [34]. In addition, DCs isolated from the mesenteric lymph nodes and Peyer's patches have been shown to favour class switching to IgA, suggesting that antigen presentation in the intestine is inherently biased towards a tolerogenic response [35, 36].

As the predominant response following oral exposure to foods is induction of immunological tolerance, much research has focused on the factors that may interfere with the development of tolerance such as age, commensal flora, and infection. Of importance, factors that affect the integrity of the intestinal epithelium may be capable of skewing the inherent tolerogenic immune response to innocuous antigens, such as peanut, by inducing inflammation that may inhibit or overcome the development of tolerance. Clinical evidence has shown a correlation between a loss of barrier integrity and the

development of food allergy [37]. As previously mentioned, the development of oral tolerance is impaired in both Crohn's Disease and Ulcerative Colitis patients, suggesting that chronic intestinal inflammation, possibly as a result of a pre-existing defect in intestinal permeability, is capable of interfering with the development of tolerance [38]. First-degree relatives of IBD patients often have increased intestinal permeability, suggesting a pre-existing defect rather than an effect of inflammation [39]. It is currently unknown whether food allergy is increased in this population of relatives. Interestingly, patients subjected to organ transplant have increased intestinal permeability due to immunosuppresing drugs (tacrolimus) and a correlative increase in IgE, and new-onset food allergies has been observed in these patients [40, 41]. While these clinical studies nurture intriguing hypotheses, they preclude conclusions concerning the role of intestinal permeability in the development of food allergy, as there are a number of confounding variables.

Tight regulation of the intestinal milieu is essential in preventing sensitization to innocuous antigens. For example, experiments in IL-10 deficient mice that spontaneously develop colitis show that while an increase in intestinal permeability precedes the development of colitis, commensal flora are pivotal, as germ free IL-10 deficient mice do not exhibit increased permeability [42]. The authors suggest that IL-10 deficiency promotes an increase in IFN-ϒ and TNF-α in the ileum and colon due to an intolerance to commensal bacteria [42]. These proinflammatory cytokines have been shown to directly disrupt epithelial barrier integrity [43, 44]. Furthermore, in a mouse model of gastrointestinal colonization with *Candida albicans*, sensitization to ovalbumin was induced as a consequence of the increased passage of soluble food antigens into circulation. The increase in intestinal permeability was dependent on the recruitment and degranulation of MCs as sensitization was entirely absent in MC deficient mice [45]. It is unclear whether increased intestinal permeability is sufficient for the induction of sensitization. Some evidence suggests that increased intestinal permeability can be detected before the development of inflammation or food allergy. As in the development of colitis, the roles of permeability, microbiota, and inflammation in disease pathogenesis may be intertwined in the development of food allergy. Thus, a significant unanswered question in peanut allergy is whether a defect in the integrity of the intestinal barrier can lead to the development of sensitization.

ANIMAL MODELS

Since the normal response to ingested food antigens is tolerance, animal models of oral sensitization rely on the administration of strong adjuvants, such as cholera toxin (CT) or staphylococcus enterotoxin B (SEB), over a period of 4-6 weeks to break or prevent the induction of oral tolerance [15, 46]. While the mechanism of these adjuvants remains under investigation, it is thought that they act on APCs to stimulate the innate immune response. Insults, such as these toxins, modulate the intestinal environment by activating immune cells through danger associated molecular pattern signaling, including Toll like receptors and Nod like receptors. Activation of these receptors ultimately results in the release of a plethora of cytokines that condition the adaptive immune response.

Cholera toxin has been shown to activate a multitude of synergistic pathways conducive to the promotion of a Th2 response. Antigen presentation by DCs, macrophages, and B cells is enhanced by increased expression of MHC II and co-stimulatory molecules, including OX40 ligand on DCs [47, 48]. Furthermore, downregulation of IL-12 and Th1 responses, as well as an increase in intestinal permeability and antigen uptake have been identified as potentially important mechanisms of cholera toxin-induced Th2 immunity [49, 50]. Moreover, IL-9 transgenic mice develop ovalbumin-induced allergy following oral administration in the absence of exogenous adjuvant, such as cholera toxin, and development of allergy is dependent on MC mediated increases in intestinal permeability [51]. Thus, a variety of intestinal insults have the potential to induce Th2 sensitization to a co-administered protein.

In accordance with the evidence that increased intestinal permeability may be linked with the development of peanut allergy, we hypothesized that the acute treatment with NSAIDs, which lead to a transient increase in intestinal permeability, may be one life-style factor that contributes to the considerable increase in food allergy over recent decades.

NSAIDS

Non-steroidal anti-inflammatory drugs (NSAIDs) are frequently used in children for the treatment of pain, fever and inflammation. It has been shown that within a given one-week period, 7.7% of American children from birth to two years of age receive ibuprofen, and 23% receive acetaminophen [52]. Furthermore, in a study of 200 children, ranging from newborn to 10 years, it was reported that 51% had received an inaccurate dose of NSAIDs, potentially much higher than recommended [53]. Treatment with NSAIDs may be associated with significant adverse effects. During chronic use, NSAID-induced enteropathy is common, usually subclinical, and can involve increased intestinal permeability, inflammation and erosion of the stomach and intestine, anemia, bleeding, perforation, obstruction and diverticulitis [54]. NSAIDs increase intestinal permeability by interfering with the integrity of the intestinal epithelium. This may enhance the passage of antigens primarily through the paracellular pathway by disrupting epithelial cell contacts, and permitting the passage of larger molecules [55].

NSAID-INDUCED ENTEROPATHY

NSAIDs act through the inhibition of cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2). Both cyclooxygenase enzymes catalyze the addition of two oxygen molecules to arachidonic acid, a 20-carbon fatty acid chain from cell membranes, forming the molecule PGH2 [56]. PGH2 is the precursor for both prostaglandins and thromboxanes, which require additional enzymes (isomerase, thromboxane synthase, and prostacyclin synthase) to continue specialization [57]. Thromboxanes are involved in vasoconstriction and aggregation of platelets. Prostaglandins act as local autocrine or paracrine messengers, inducing smooth muscle contraction and dilation of vascular and

bronchial smooth muscle, aggregation and disaggregation of platelets, and regulation of inflammation [57].

COX-1 is constitutively active in the stomach, intestine, kidneys and platelets. It is responsible for the conversion of arachidonic acid to PGE2, TxA2, and PGI2. PGE2 exerts gastroprotective effects by signaling through the EP3 receptor to decrease gastric acid secretion, increase gastric mucus secretion, and induces contraction of GI smooth muscle (also uses the EP1 receptor). It functions through the EP2 receptor to induce relaxation of GI smooth muscle [57, 58].

COX-2 is constitutively active in the intestine, but is otherwise induced at sites of inflammation by macrophages, endothelial cells, T cells and synovocytes, where it facilitates the production of inflammatory PGs, PGE2, proteases and reactive oxygen species [57, 58]. In the intestine, COX-2 catalyzes the production of PGE2, which protects the intestine through increasing mucus production, and has been shown to influence the induction of Tregs through induction of FoxP3 in naïve $CD4^+$ T cells [59]. Furthermore, treatment with selective COX-2 inhibitors has been shown to result in an increase of intestinal IL-4 producing T cells and a correlating decrease in the number of MLN Tregs. In this system, COX-2 expression was shown to be necessary to induce a TGF-β and retinoic acid-dependent induction of FoxP3 in naïve T cells. Importantly, COX-2 deficiency caused functional impairment of tolerance (assessed by changes in delayed type hypersensitivity) to orally delivered proteins (OVA), which could be overcome by treatment with an IL-4 neutralizing antibody during protein feed [60-62].

Most NSAIDs are non-selective inhibitors of both COX enzymes and result in GI side effects through the inhibition of gastro-protective prostaglandins. Intestinal damage affects 70-80% of patients taking NSAIDs long term and, during acute use, as is common in children, damage is usually subclinical [63]. Increased intestinal permeability can be observed in patients as soon as 12 hours following the ingestion of a single dose, and is maintained for another 12 hours [64]. Increased intestinal permeability has been identified as the first step in NSAID-induced injury, allowing for translocation of intestinal bacteria, pancreatic secretions, and food antigens across the epithelium, resulting in activation of phagocytes and APCs, and recruitment of other inflammatory cells [58, 65]. Inflammation and intestinal damage are significantly reduced in both germ free and antibiotic treated animals exposed to NSAIDs, suggesting that exposure to commensal bacteria plays a significant role in NSAID-induced intestinal damage [66, 67].

EPICUTANEOUS SENSITIZATION

CLINICAL EVIDENCE AND RATIONALE

Skin provides a physical barrier between the environment and internal systems. The skin is home to both structural and immune cells that are capable of dynamically responding to a plethora of insults, as well as maintaining homeostasis under normal conditions. Unlike the gastrointestinal tract, which is exposed to an immense number of novel food antigens, the skin is much less permissive to the entry of antigens, as the outermost layers of the epidermis provide a barrier that is highly restrictive to the passage

of most antigens and bacteria [68]. Consequently, breaches in the epidermis rapidly trigger the release of inflammatory cytokines, followed by activation of the epidermal resident APC, Langerhans cells (LCs), and subsequently, the initiation of an adaptive immune response [69] [70].

The relationship between peanut exposure through the skin and the development of peanut allergy gained significant attention following a 2003 paper in the New England Journal of Medicine by Lack *et. al*., which analyzed data from the Avon Longitudinal Study of Parents and Children. This study showed that children with skin barrier disruptions (defined as rash over joints and skin creases or oozing crusted rashes) who were exposed to skin creams containing peanut oil, were at a significantly greater risk of developing PA than normal or atopic control subjects. Indeed, it was found that 91% of peanut allergic children in the cohort had been exposed to peanut oil containing skin creams during infancy, whereas only 53% of atopic controls and 59% of normal controls had been exposed to similar skin preparations. This study hypothesized that exposure to low levels of peanut protein and peptides that have been confirmed to be present in refined peanut oil, through inflamed skin could result in sensitization on its own, or through an adjuvant effect of the oil [11] [71].

Following this initial study, a correlation was noted between loss-of-function mutations in the fillagrin gene, which encodes a protein that is essential in forming the *stratum corneum* (outermost layer of the epidermis that acts as a permeability barrier), with the incidence of peanut allergy. Interestingly, mutations of the fillagrin gene were found in approximately 20%, of peanut allergic patients in three separate cohorts of English, Dutch, and Canadian patients, compared with 5-11% of control subjects. This correlation persisted once confounding factors, such as atopic dermatitis, were taken into account [12].

Together, these studies provide strong clinical evidence to support the hypothesis that a skin barrier defect is a significant risk factor in the development of peanut allergy. Based on these initial clinical studies, a number of animal models have been established to determine whether exposure to protein antigens through the skin is capable of inducing allergy and, to a lesser extent, study the mechanisms that play a role in epicutaneous sensitization.

PHYSIOLOGY OF THE EPIDERMIS

The skin is comprised of two major layers, the epidermis and the dermis. The outermost layer of the skin functions as an interface between the inner and outer environment, and is called the epidermis. [72, 73]. The epidermis consists of 5 discrete cell layers that are distinguished by the differentiation status of the keratinocytes. The bottommost layer, the *stratum basale* borders the underlying dermis, and lies below the *stratum granulosum* (distinguished by the granular pockets of lipid in the keratinocytes), *stratum spinosum*, *stratum lucidum*, and the outermost *stratum corneum*. Stem cells originate in the *stratum basale*, and move through the layers towards the skin surface as they differentiate. As cells move from the *stratum spinusum* to the *stratum corneum*, they excrete fatty acids that are stored in granules within the keratinocytes. These fatty acids

fill the spaces between the now highly keratinized, terminally differentiated keratinocytes, and form a permeability barrier. A frequently used analogy for the *stratum corneum* composition is a brick-and-mortar construction, where the keratinocytes are the bricks and the fatty acids are the mortar [74, 75]. Consequently, disruption of either the keratinocytes by mechanical injury, or the fatty acid constituents, through FA deficient diet or solvents, may lead to the increased entry of environmental antigens and loss of water from the skin [69].

IMMUNOLOGY OF THE EPIDERMIS

KERATINOCYTES

As with the epithelial cells of the intestine, keratinocytes are the first cells to contact allergens, and may participate in immune responses as either non-professional APCs, or (primarily) through the production of pro-inflammatory cytokines that condition professional APCs, such as Langerhans cells. Keratinocytes have been shown to produce cytokines, including IL-1α, IL-1β, and TNF-α [76, 77]. IL-10 is produced constitutively by keratinocytes and is upregulated following exposure to irritants, including contact sensitizers, and UV radiation [78, 79]. Thus, as with intestinal epithelial cells, keratinocytes have the capacity to condition the immune response depending on the nature of the stimuli. As APCs, keratinocytes produce little IL-12p40, and as a result, activate T cells to preferentially produce IL-2 and IL-4, but not IFN-γ [80].

LANGERHANS CELLS

Langerhans cells are the epidermal resident subset of dendritic cells, and constitute the only professional antigen presenting cell subset found in the steady-state epidermis. These cells are long lived, and persist for weeks to months in the normal epidermis [81]. Langerhans cells establish an evenly distributed network throughout the epidermis by extending their dendrites to overlap with those of adjacent cells. Naïve LCs have high phagocytic and pinocytic capacities, as well as the ability to efficiently process protein antigens. *In vitro* treatment of LCs with GM-CSF has been shown to induce maturation of LCs [81, 82]. Following activation through signals from other cells and danger associated molecular pattern signaling, LCs begin to mature and substantially increase MHC-II expression, costimulatory molecules CD80/86 and CD40, and also lose much of their ability to phagocytose. As early as 2 hours following application of peanut protein to tape stripped ear skin, LCs change their morphology and begin to migrate to draining lymph nodes, carrying the peanut protein. [70].

THE DERMIS

The dermis contains an armada of immune cells that are well situated to initiate a rapid immune response following injury or infection. There are three major subsets of DCs present in the normal dermis: langerin$^+$ dermal DCs, langerin$^-$ dermal DCs, and LCs in transit from the epidermis to the lymph node [81]. The functional roles of the langerin$^+$

and langerin$^-$ dermal DCs have not yet been fully elucidated. The development of conditional knockout mice, as well as the differential rates of repopulation of these cell subsets following depletion of langerin$^+$ cells in a DTR model is beginning to shed light on the distinct roles of these subsets [83]. However, a lack of consensus in identifying these cells during migration, as well as an inability to replicate some studies bring more questions than answers.

Given the imminent threat that a breach in barrier poses, and the plethora of environmental antigens and pathogens that may access the skin, a large pool of memory T cells are concentrated in ideal locations to mount a rapid recall response. It has been estimated that 20 billion (2x more than in peripheral circulation) T cells are present in normal adult skin. Of these, >95% are memory T cells, and less than 5% are naïve. In addition, a large number of regulatory cells are positioned ideally to downregulate inflammatory responses, both following infection or injury, as well as in the steady state, which is essential for the prevention of autoimmunity [84]. Indeed, 5-10% of all cutaneous T cells are CD25$^+$ FOXP3$^+$ regulatory T cells, capable of local proliferation during inflammation [85, 86]. In addition to these major cell types, skin-resident plasmacytoid DCs, fibroblasts, and NKT cells have been shown to play important roles in some infection models [72].

ANIMAL MODELS:

A number of procedures have proven effective in facilitating epicutaneous sensitization, all of which alter the integrity of the *stratum corneum*. The best-studied protocol uses a procedure termed tape stripping, in which a piece of cellophane tape is used to remove the *stratum corneum* on both sides of the ear. This is followed by application of antigen using a cotton swab. Studies using this method of sensitization have shown that epicutaneous exposure to peanut prevents the subsequent development of high dose oral tolerance [87]. Indeed, following epicutaneous exposure, feeding peanut did not induce tolerance-associated reductions in delayed type hypersensitivity and *in vitro* T cell proliferation, and instead induced both Th2 cytokine and immunoglobulin responses [87]. This model induces sensitization with only three consecutive applications of antigen; however, it does not induce anaphylaxis.

The second procedure that has been effective in epicutaneously sensitizing animals to food antigens relies on application of an antigen soaked pad, secured under an occlusive patch for several weeklong intervals. Application of ovalbumin, cashew, or hazelnut protein under these conditions is capable of inducing robust sensitization and anaphylaxis following systemic antigen challenge [88] [89] [90].

Fillagrin deficient mice, which have impaired barrier function, spontaneously develop skin inflammation and increased levels of total IgE after 28 weeks of life. Epicutaneous exposure to ovalbumin (OVA) in these mice results in allergic sensitization, shown by increased OVA-specific IgG1 and IgE. [91]. These studies all stress the potential role of epidermal dysfunction in the development of allergy.

Comparing the effects of three methods of barrier disruption: tape stripping, acetone disruption of *stratum corneum* lipid components and diet-induced fatty acid deficiency, Wood *et al.* found that pro-inflammatory cytokine mRNA expression was universally increased. Indeed, TNF-α, IL-1α, IL-1β, and GM-CSF were shown to increase several fold over control mice [92]. Thus, a number of methods of skin barrier disruption seem to lead to a similar pattern of pro-inflammatory cytokine expression, with the capability to induce LC maturation and migration and sensitization to proteins.

NEONATAL IMMUNITY

The immune system undergoes considerable development during infancy in order to accommodate the shift between the relatively sterile intrauterine life, where protection from autoimmunity is essential, and infancy, where colonization and immunoprotection become of paramount importance. As peanut allergy develops early in life, it would seem intuitive to investigate the etiology of peanut sensitization in neonatal models.

NEONATAL INNATE IMMUNITY

Neonatal skin undergoes significant immunological development during the first two weeks following birth. Protection from infection and maintenance of bacterial colonization are controlled by the increased expression of antimicrobial peptides in the epidermis [93]. One cell compartment that undergoes particularly significant changes during the first two weeks of life in the mouse is the cutaneous APC network of the skin (Langerhans cells (LCs) and dermal dendritic cells). Over this period of time, LCs in the epidermis mature from a globular cell shape into cell bodies with well defined dendrites that extend in an overlapping net throughout the epidermis and, furthermore, increase their ability to present antigen and migrate to draining lymph nodes [94, 95].

NEONATAL ADAPTIVE IMMUNITY

T CELLS

Historically, the neonate has been deemed incapable of mounting an adaptive immune response. Pioneering work in neonatal skin by Billingham and Medawar showed that neonatal mice were able to tolerate skin grafts from immunologically distinct hosts, and moreover, injection of adult splenocytes during infancy resulted in a lifelong tolerance to those cells [96, 97]. These experiments led to the prevailing hypothesis of "neonatal tolerance," where the default neonatal response to foreign antigens was thought to be tolerance. Subsequently, based on studies showing that neonates frequently responded to infections with an inappropriate Th2 response, it was suggested that the neonatal T cell response is intrinsically skewed towards a Th2 phenotype [98]. However, studies have shown that depending on the stimuli, neonates can produce adult levels of either Th1 or Th2 type responses [99]. Limited IL-2 production in neonates restricts

proliferation of T cells and, indeed, neonatal mice have far fewer T cells than adults [100]. However, despite the reduced number of T cells in the neonate, the ability to mount adult level immune responses suggests that the T cell compartment is not inherently deficient.

B CELLS/IMMUNOGLOBULINS

Neonatal B cell responses are fundamentally different from adult responses. More time to achieve peak levels, and lower peak antibody levels are characteristic of the neonatal immunoglobulin response [100]. This is likely due to the fact that neonates have a greater number of immature B cells in the spleen ($IgM^{+}IgD^{-}$), that do not upregulate co-stimulatory molecules and MHC class II to maximize interactions with T cells. Neonatal lymph node B cells tend to show greater maturity than B cells located in the spleen. However, the ability to form germinal centers, where B and T cells come into contact, is not achieved until 3 weeks of age in mice, and 4 months in humans [100]. It has been shown that under certain conditions, neonates are capable of producing adult level immunoglobulin responses. For example, Jakobsen *et al.* showed that intranasal, but not parenteral, delivery of a pneumococcal polysaccharide conjugate vaccine was able to induce protective immunity in neonatal mice, suggesting that the location of priming affects the immunoglobulin response in neonates [101]. However, as neonatal germinal center formation occurs over the first few days to weeks following life, induction of a strong B cell response is difficult before this maturation is complete [102-104]. Adoptive transfer of neonatal splenocytes to adult SCID mice resulted in the formation of lymphoid follicles, but transfer of adult B cells to neonates could not induce germinal center formation. Therefore, it has been suggested that follicular DC immaturity is a limiting factor in neonatal B cell responses [105].

RATIONALE

This study aims to address the impact of barrier integrity first in the GI system, and second, in the skin in the development of PA. The investigation of the second aim included studies in adulthood as well as early in life as potential causative factors of PA. To investigate the first goal, we evaluated the impact of non-steroidal anti-inflammatory drug (NSAID)-induced gastrointestinal damage in facilitating allergic sensitization. NSAIDs increase permeability in both the stomach and intestine, thus permitting access of greater numbers of food proteins as well as the ubiquitous commensal flora into the immune-protected areas of the intestine, leading to an inflammatory response. We hypothesized that in such an environment, sensitization may occur to orally administered peanut in the absence of adjuvant. The second aim of this study was to investigate the role of skin barrier disruption in eliciting sensitization to peanut. In contrast to the GI tract, the skin is a non-mucosal site that maintains a large number of immunocompetent cells that are primed to react to barrier-disruption. We hypothesized that exposure to peanut through disrupted barrier skin would result in the development of peanut allergy.

We suggest that studies examining the interaction between disrupted mucosal (GI) and non-mucosal (skin) barriers, in concert with the impact of immunological development (age) are crucial to understanding and preventing the increasing prevalence of PA and, more importantly, bringing about solid guidelines for safe peanut exposure in children.

OBJECTIVES

This project consists of three major objectives. First, we aim to determine whether oral exposure to peanut during NSAID-induced intestinal pathology is capable of bringing about sensitization. Second, we aim to develop an epicutaneous model of peanut sensitization in adult mice that leads to robust PA. Finally, we aim to determine the relevance of epicutaneous sensitization early in life.

CHAPTER 2: MATERIALS AND METHODS

Mice. 6-8 week old C57BL/6 and BALB/c breeding pairs were purchased from Charles River (CR) (Ottawa, ON, Canada). 6-8 week old C3H/HeJ breeding pairs were purchased from Jackson Laboratories (JAX) (Bar Harbor, Maine, USA). All indomethacin experiments were carried out in C57BL/6 females from CR. All epicutaneous experiments used 6-8 week old male and female mice from CR or JAX (C3H/HeJ only) and, when available, from our breeding colonies. Mice were housed under SPF conditions on a 12 hour light-dark cycle. Breeding pairs were housed in microisolator cages, with opaque dividers between cages, and fed a high fat diet at McMaster University in the Central Animal Facility. Newborn mice were housed with their parents, and weaned at 3 weeks of age.

Peanut. Defatted Crude Peanut Extract (Greer, Lenoir, NE) was reconstituted in sterile PBS to a final concentration of either 20 mg protein/ml (for epicutaneous sensitization), or 7.64 mg protein/ml (for intraperitoneal challenge). Kraft All Natural™ peanut butter (1 mg protein/3.84 mg weight) was diluted in PBS for oral sensitizations.

Oral Sensitization Model (Peanut and Cholera Toxin). Mice were given 5 µg of Cholera Toxin (CT) and 3.84 mg of peanut butter in a total volume of 500 µl of sterile PBS by gastric gavage for 4, 7 or 10 consecutive days. Mice were rested for 2 weeks and then challenged intraperitoneally (i.p) with 3.82 mg CPE. Mice were monitored for 40 minutes for signs of anaphylaxis, which were scored as: 1. Scratching in ear, 2. Reduced motion/self isolation, 3. Motionless/lying prone, 4. No response to whisker stimulus, 5. Endpoint, seizure/death. Rectal temperature was taken using a digital thermometer (VWR, Mississauga, ON), and was recorded every 10 minutes. At 40 minutes, ~100 µl of blood was collected from the retroorbital plexus using a heparin coated microhematocrit capillary tube, and hematocrit was measured using a hemastat microhematocrit centrifuge (Separation Technology Inc., Stanford, FL). Mice were given 1 ml of warm PBS

subcutaneously (s.c.), and allowed to rest for 72 hours before collection of tissues for culture and peritoneal lavage.

Indomethacin. Indomethacin was purchased from Cayman Chemical (Ann Arbor, MI). The drug was prepared fresh daily and dissolved by slowly heating in a 5% solution of Na_2CO_3 and adjusted to a pH of 7.4. Mice were divided into two groups, one receiving 5 mg/kg given every second day, and the other receiving 3.5 mg/kg daily for 10 days by gastric gavage. Mice in both groups were given 1 mg of peanut protein from peanut butter in 500 µl PBS daily by gastric gavage.

Epicutaneous Sensitization (long protocol, 42 days). A 1-centimeter (cm) by 1 cm patch of dorsal skin, beginning 0.5 cm above the tail, was shaved using an electric razor (Wahl, Sterling IL). 24 hours later, a piece of cellophane tape (Grand and Toy) was evenly applied, and then immediately removed from the exposed patch of skin 7-9 times (until the skin had a light sheen, and removed section of tape was not cloudy). Alternatively, using the ear as a site of sensitization, mice were anesthetized using isofluorine, and each side of the ear was tape-stripped 7-9 times. 200 µg of CPE in 10 µl of sterile PBS was immediately applied to the tape-stripped skin, and allowed to dry before mice were returned to their cages. CPE application was repeated for 10 consecutive days, and tape stripping was performed on alternating days. Mice were exposed to this tape-stripping and peanut routine for 3 one-week intervals, each separated by one week of rest (figure). Following the final CPE exposure, mice were rested for 7 days, and then serum was collected from the retroorbital plexus for antibody analysis. 24 hours later, mice were challenged i.p. with 3.82 mg of CPE in 500 µl of PBS to assess anaphylactic responses.

Epicutaneous Sensitization/Exposure (short protocol, 7 or 10 days). Adult and neonatal mice were exposed to either 7 or 10 consecutive days of CPE application to shaved (excluding newborns) and tape-stripped skin, followed by a 3 week rest period. Neonatal mice were exposed to this protocol beginning at either 1 day following birth, or at 2 weeks of age for a 10-day period. Parents were moved to a separate cage during neonatal manipulations. Mice exposed beginning at 1 day were tape-stripped 5-7 times, and 200 µl of peanut protein 10 µl of PBS was applied to the tape-stripped area and allowed to dry before returning the parents. Tape stripping was performed daily in this group, as cell turnover is much higher than adult mice [106]. Shaving was not necessary until between days 6 and 8 of life, and any fur that began to grow was removed using a mechanical razor (Gilette). 2-week-old mice were shaved and treated the same as the newborn group. Mice were rested for 3 weeks, at which time serum antibodies were analyzed, followed by i.p. CPE challenge.

Peritoneal Lavage. 72 hours following CPE challenge i.p., mice were anesthetized with isofluorine, and the abdomen was soaked with ethanol. 3 ml of 5 mM PBS-EDTA was injected intraperitoneally, and the abdomen was firmly massaged for one minute. Peritoneal lavage fluid was extracted through a small opening using a 1 ml pipette, and

immediately stored on ice. Lavage fluid was centrifuged at 4°C at 1200 RPM for 10 minutes. Supernatant was poured off, and the pelleted cells were resusupended in 1 ml cold PBS. Samples were diluted to $1.65x10^5$ cells/ml. Cytospins of the cells were stained 24 hours later with the Hema 3 stain set (Fischer scientific). A minimum of 500 cells was counted to quantify percentages of mast cells, mononuclear cells, neutrophils, and eosinophils.

Histology. Skin and intestinal segments were collected and fixed in 10% formalin for 24 hours and then stored in 70% ethanol. Sections were stained with H&E for microscopic analysis.

Measurement of Peanut-specific Immunoglobulins:

IgG1 and IgG2a. Peripheral blood was collected by bleeding from the retroorbital plexus at specified time points following the last peanut exposure. Serum levels of peanut-specific IgG1 and IgG2a were measured by ELISA. 96-well plate Maxi-Sorp plates (Nunc; VWR) were coated with 2 μg/ml CPE (Greer) in 50 nM carbonate-bicarbonate buffer (pH 9.6; Sigma-Aldrich) at 4°C, and incubated overnight. The following morning, coated plates were blocked with 1% BSA/PBS for 2 hours at room temperature. Plates were washed 3 times and serum samples were incubated overnight at 4°C. Samples were diluted as follows: for IgG1 detection 1/20, 1/200, ½,000, and 1/20,000 and for IgG2a detection ½, 1/20, 1/100, and 1/500. The reactions were developed with biotinylated goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates), respectively for 2 hours at room temperature. Plates were washed and incubated with alkaline-phosphatase streptavidin (Sigma-Aldrich) for 1 h at room temperature. The color reaction was developed with *p*-nitrophenyl phosphate tablets and stopped with 2 M H_2SO_4.

Peanut-specific IgE: Serum peanut-specific IgE was measured by sandwich ELISA. 96-well plate Maxi-Sorp plates (Nunc; VWR Canlab) were coated with 2 μg/ml purified rat anti-mouse IgE Ab (BD Pharmingen) in PBS overnight at 4°C. Coated plates were washed and blocked with 10% normal serum 1% BSA/PBS/0.5% Tween 20 for 1 h at 37°C. After washing, serum samples (four serial dilutions) were incubated for 2–3 h at room temperature. Each sample was diluted 1:2. Subsequently, a CPE-digoxigenin (DIG) conjugate solution was added (1 h at 37°C). The coupling of DIG to CPE was performed according to the manufacturer's instructions (Roche). After incubation (1 h at 37°C) with peroxidase-conjugated anti-DIG fragments (Roche), a tetramethylbenzidine substrate (0.1 mg/ml) solution was used and the color reaction was stopped with 2 M H_2SO_4. Absorbance was measured at 450 nm and results expressed as nanograms of DIG-CPE bound times the dilution factor.

CHAPTER 3: ORAL SENSITIZATION AND THE IMPACT OF INDOMETHACIN

SHORTENED MODEL OF ORAL SENSITIZATION

Sensitization to peanut may occur during the course of an acute intervention, such as during a GI infection or treatment with antibiotics or other drugs that interfere with mucosal integrity, such as the pervasively used NSAIDs. Traditional models of sensitization to peanut are useful for the investigation of mechanisms of anaphylaxis but are limited in application to investigation of these short-term interventions due to the lengthy duration required to induce peanut-specific immunoglobulins. Therefore, the development of a shorter model, in which sensitization occurs following a short period of exposure was essential. To test whether a series of consecutive gavages with peanut and cholera toxin (CT) could induce sensitization, C57BL/6 mice were gavaged daily for 4, 7 or 10 days with 5 μg of CT and 3.82 mg of peanut butter (Figure 2a). Following a two-week rest period, serum immunoglobulins were measured. Mice that received 4 consecutive gavages (the number of gavages in traditional models of oral sensitization) did not develop significant levels of either peanut-specific IgE or IgG1 (Figure 2 b,c). However, mice that received either 7 or 10 gavages developed significant levels of peanut-specific IgG1, as well as slightly, but not statistically significantly increased levels of peanut-specific IgE (Figure 2 b,c).

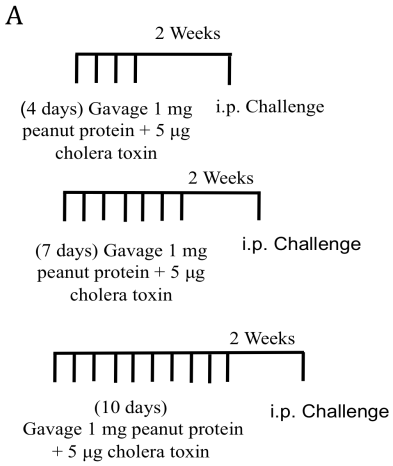

Figure 2 a: Schematic of Oral Sensitization Protocols. Oral administration of peanut and cholera toxin for 4, 7, or 10 consecutive days in C57BL/6 mice

Mice that received only 4 exposures did not develop significant signs of anaphylaxis (clinical score of 0), hypothermia, or hemoconcentration following systemic (i.p.) challenge with crude peanut extract (CPE) (Figure 1 d-f). In contrast, both the 7 and 10-day exposure groups developed severe anaphylaxis (clinical scores between 2-3) and hypothermia 40 minutes following challenge (4-5^{o}C decrease in temperature) (Figure 1 d,e). Hemoconcentration in both the 7 and 10-day sensitization groups was similar, with significantly increased hematocrit levels to approximately 55% (Figure 1 f). In initial

experiments, both 7 and 10 days of exposure resulted in 80% of mice becoming sensitized. However, in repeat experiments, 7 days of exposure resulted in approximately 50% of mice becoming sensitized, whereas 10 consecutive gavages maintained a consistent 80% sensitization rate (data not shown). Therefore, as 10 consecutive days of peanut and cholera toxin feed by gastric gavage results in consistent and robust allergic sensitization; this model was chosen for future experiments involving administration of indomethacin during sensitization.

In contrast to a traditional model of sensitization (gavage of peanut and cholera toxin once a week for four weeks, followed by two weeks of rest), clinical signs of anaphylaxis are less pronounced in a 10-day model (3-5 and 3-4 respectively). Hypothermia is also slightly less severe, with an average decrease to 29°C in conventional models compared to 32°C in a 10-day model. Peanut-specific IgE, which is increased in conventional models is not significantly increased in the 10 day model. In contrast, hematocrit and peanut-specific IgG1 are similarly increased in both models [15].

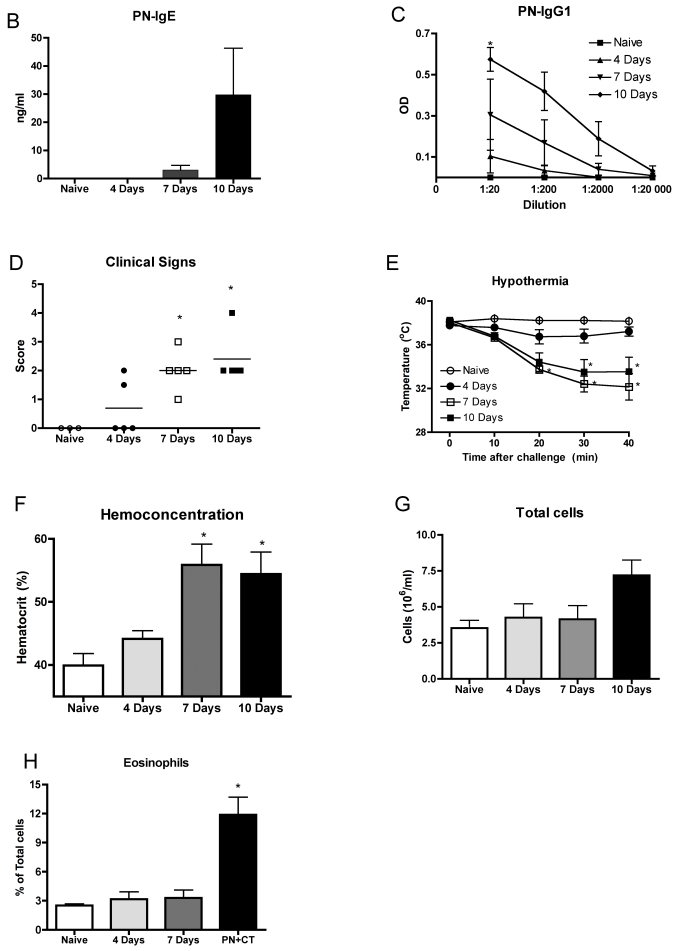

Figure 2 b-h: Shortened Model of Oral Sensitization. Levels of PN-specific IgE (b) and IgG1 (c) in C57BL/6 mice 2 weeks after 4, 7, or 10 consecutive oral exposures. C57BBL/6 mice were gavaged with 1 mg of peanut protein and 5 µg of CT. Mice were challenged i.p. with 3.82 mg of peanut protein and monitored for 40 minutes for clinical signs of anaphylaxis (d), and core body temperature (e).

Hematocrit was taken at 40 minutes after challenge (f). Total cell number (g) in peritoneal lavage was evaluated by counting 500 cells under 40x magnification. Percentage of eosinophils in peritoneal lavage (h) was also evaluated. Data presented as m ± SEM, n=3-7 mice per group. *p<0.05.

ROLE OF NSAIDS IN PEANUT ALLERGY

ROLE OF INDOMETHACIN IN SENSITIZATION

First, a dose-response study was performed to determine the maximum tolerated oral dose of indomethacin. The oral LD50 reported by the manufacturer for use in mice is 11.841 mg/kg. Because drug administration was required daily, we chose a maximum dose of 7.5 mg/kg, which had been previously used successfully to increase intestinal permeability in rat single dose experiments, when delivered subcutaneously [107]**.** A dose of 3.5 mg/kg of indomethacin delivered orally in mice has been shown to increase intestinal permeability without producing macroscopic or microscopic ulcers when given 3 times per week [55]. Therefore, we chose to administer this dose daily, for 10 days. A dose of 5 mg/kg was also chosen as an intermediate between the two extreme doses (Figure 3 a). We found that 7.5 mg/kg resulted in 100% mortality after the second gavage, 5 mg/kg was tolerated on alternating days (10% mortality), and 3.5 mg/kg was tolerated daily (10% mortality) (data not shown). Following 10 days of exposure, jejunal segments from the group treated with 3.5 mg/kg indomethacin were similar to the naïve group, whereas the groups that received cholera toxin or 5 mg/kg showed decreased villus height, showing that 3.5 mg/kg of indomethacin had minimal effects on intestinal integrity; however, 5 mg/kg produced some intestinal damage (Figure 3 b).

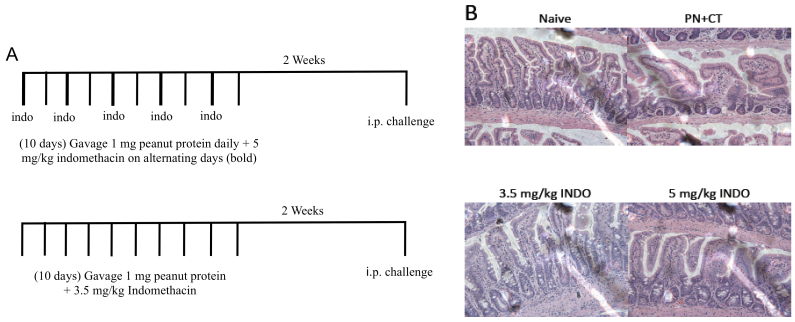

Figure 3 a-b: Schematic for Indomethacin Treatment and Intestinal Histology. Schematic for indomethacin and peanut oral administration (a) and intestinal histology following peanut and cholera toxin, 3.5 mg/kg indomethacin, and 5 mg/kg indomethacin (b).

Following the dose-response experiment, two doses were chosen. Both groups received indomethacin daily, by oral gavage. One group of mice was administered 5 mg/kg of indomethacin on alternating days, and a second group was given 3.5 mg/kg daily. In all instances 3.75 mg of peanut butter was gavaged daily, without cholera toxin (Figure 3 a). Peanut-specific IgE and IgG1 were measured from serum obtained 2 weeks following peanut and indomethacin exposures. Neither the 3.5 mg/kg nor the 5 mg/kg groups developed peanut-specific IgE (Figure 3 c). The 3.5 mg/kg group showed sporadic

levels of peanut-specific IgG1 (1/5 mice) that did not reach statistical significance. The 5 mg/kg group did not develop any peanut-specific IgG1 (Figure 3 d).

Neither indomethacin treated group showed clinical signs of anaphylaxis following systemic challenge (Figure 3 e). Likewise, temperature and hematocrit values were not significantly different from naïve mice (Figure 3 f,g). 72 hours after challenge, the late phase response was evaluated. Indomethacin treated mice did not show an increase in total cell number in the peritoneal lavage, or an increase in the percentage of eosinophils in the PL (Figure 3 h,i).

Thus, over a 10-day course of indomethacin treatment, administration of peanut did not result in peanut sensitization or anaphylaxis. Indomethacin is no longer recommended for use in children, but was previously suggested at a dose not greater than 2 mg/kg per day, and a maximum dose of 200 mg is recommended for adults (ranging from 2-3 mg/kg). As the doses of indomethacin used in this study exceed the maximum recommended dose for use as an NSAID in humans, our findings would suggest that it is unlikely that NSAIDs, such as indomethacin, play a role in sensitization to peanut in children following acute use and appropriate dosage.

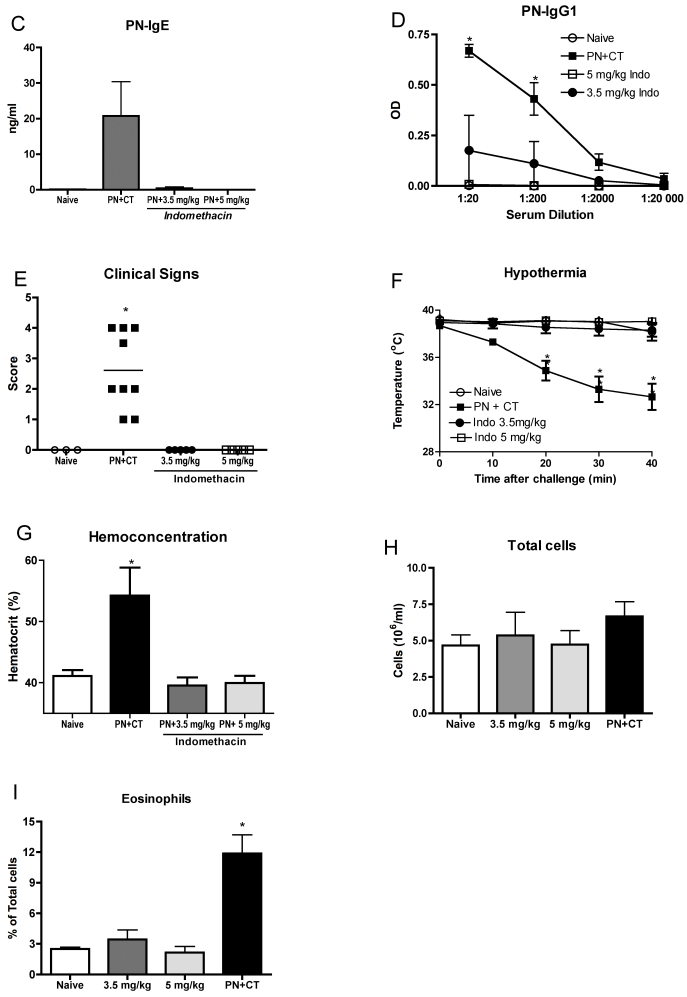

Figure 3 c-i: Role of Indomethacin in Oral Sensitization. Mice were exposed to peanut by gavage during oral indomethacin treatment for 10 consecutive days. Serum levels of PN-specific IgE (c) and IgG1 (d) were analyzed 2 weeks following the final exposure. Peanut and indomethacin exposed mice were challenged i.p. with 3.82 mg peanut protein and monitored for signs of anaphylaxis. Clinical signs of anaphylaxis (e), core body temperature (f), and hematocrit 40 minutes after challenge (g) were evaluated. Total cell number (h) in peritoneal lavage was evaluated by counting 500 cells under 40X magnification. Number of eosinophils in peritoneal lavage (i) was also evaluated. n=3-8 mice. Data presented as m±SEM. * $p<0.05$

ROLE OF INDOMETHACIN BEFORE CHALLENGE

Our next avenue of investigation was to determine whether NSAID treatment before oral challenge in peanut-sensitized mice would facilitate anaphylaxis. Mice were sensitized using the 10-day peanut and cholera toxin oral sensitization protocol, and were then given two 5 mg/kg doses of indomethacin by gastric gavage, one 24 hours before oral challenge and the second at 1 hour before oral challenge. During oral challenge mice were given an initial dose of 40 mg of peanut protein by gavage, followed by an

additional 70 mg by gavage 20 minutes after the first dose if no response (clinical score or decrease in body temperature) was observed within the first 20 minutes.

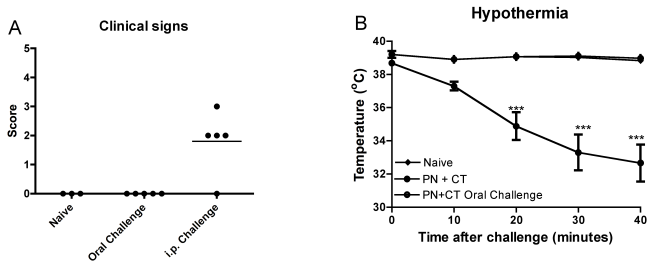

Figure 4: Role of Indomethacin in Oral Challenge. Hypothermia (a) and clinical signs of anaphylaxis (b) following oral or i.p. peanut challenge in sensitized C57BL/6 mice. Data presented as m±SEM, n=3-8 mice per group. *p<0.05

Sensitization was confirmed by analyzing serum IgG1 by ELISA (appendix A-1). Following oral challenge with the maximum dose (70 mg), no mice showed any clinical signs of anaphylaxis (Figure 4 a). Furthermore, orally challenged mice did not develop hypothermia following challenge (Figure 4 b). 40 minutes following oral challenge with the highest dose, mice were challenged i.p. to confirm sensitization. All mice showed significant clinical signs of anaphylaxis (score of 3), marked hypothermia (7^{o}C decrease in temperature), and increased vascular permeability (hematocrit 50%) (A-2). In contrast, sensitized mice that were initially challenged i.p. progressed to reduced motion or motionless by 40 minutes following challenge, and had an average clinical score of 2-3 (Figure 3 a). Temperature decreased by approximately 7^{o}C, and hematocrit was significantly increased following i.p. challenge (Figure 3 f,g). Thus, increasing intestinal permeability using indomethacin does not facilitate the development of anaphylaxis following oral challenge in peanut sensitized mice.

CHAPTER 4: ESTABLISHING A MODEL OF EPICUTANEOUS SENSITIZATION TO PEANUT

ROLE OF SITE OF PEANUT ADMINISTRATION

Our first goal was to establish a reliable model of epicutaneous sensitization in adult mice, with the potential of being applied in neonatal mice. We first compared application of peanut protein to two different skin sites, the back and the ear, to determine whether variation in site of exposure would alter peanut sensitization and anaphylaxis. As established models of epicutaneous sensitization administer protein via tape stripped ear skin or an occlusive patch on the back, our goal was to achieve sensitization through tape-stripped dorsal skin, which is a more practical route of administration for future neonatal studies. A one square centimeter patch of fur was shaved approximately 0.5 cm above the base of the tail, and this area was tape stripped using cellophane tape. Alternatively, each side of the ear was tape stripped. In both groups of mice, 200 µg of peanut protein in 100 µl of sterile PBS was applied to the tape stripped area for 7 consecutive days, followed by

a week of rest. This protocol was repeated 3 times, for a total of 3 weeks of peanut exposure (Figure 4a).

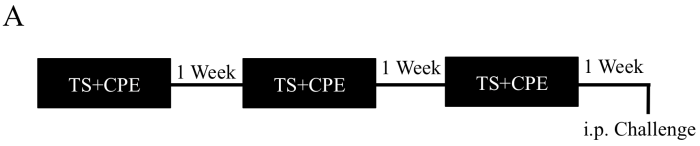

Figure 5 a: Schematic Outline of 6 week Epicutaneous Sensitization Protocol. C57BL/6 mice were treated either by shaving and tape stripping a 1 by 1 cm patch of dorsal skin, or by tape stripping both sides of one ear 8 times. 200 µg of peanut protein in 10 µl of PBS was applied to the treated area for 7 consecutive days, followed by a one week rest period. This was repeated a total of three times, at which time mice were challenged i.p. with CPE.

One week following the last epicutaneous exposure, levels of peanut-specific immunoglobulins, IgG1, IgG2a, and IgE were analyzed by ELISA in serum collected from the retro-orbital plexus (5 b-d). Significantly elevated levels of the Th2-type peanut-specific immunoglobulin IgG1 were found in both groups (Figure 5 b). Whereas there was a trend towards increased peanut-specific IgE in both groups, these levels were not statistically significant (Figure 5 c). Levels of the Th1-type peanut-specific IgG2a were not increased significantly (Figure 5 d). These data suggest that epicutaneous exposure to peanut through tape-stripped skin, either ear or back, induces predominantly Th2 sensitization.

Following blood collection, mice recovered for 24 hours, and then were challenged i.p. with 3.82 mg of CPE, and monitored for a period of 40 minutes. Mice that were sensitized to peanut through either location (ear, back) showed significant signs of anaphylaxis (clinical score of 2-5) (Figure 5 e). In fact, 66% of mice sensitized through dorsal skin and 33% of mice sensitized through ear skin reached endpoint (seizures/death). Mice sensitized through the back showed an average score of 5, whereas mice sensitized through ear skin had an average clinical score of 2.5.

Both groups of sensitized mice developed significantly decreased body temperature (up to 6 degrees Celsius) (Figure 5 f). 40 minutes following CPE challenge, both groups of sensitized mice developed significantly increased hematocrit (Figure 5 g), compared to naïve mice. Mice sensitized through the back had an average increase in hematocrit to 62%, while the ear-sensitized group had a slightly lower average hematocrit of 58%, compared to 44% for the naïve group.

72 hours following peanut challenge, the late-phase response was evaluated. Recruitment of eosinophils and the total cell number in peritoneal lavage fluid, which has been shown to peak at 72 hours in a model of oral peanut sensitization, was increased in both epicutaneously-sensitized groups over the naïve group; however, these increases were not statistically significant (Figure 5 h,i). It is likely that the inability to achieve statistical significance was due to a larger variability (SEM) and less robust sensitization compared to traditional oral sensitization models. Comparable levels of peanut-specific immunoglobulins and parameters of anaphylaxis in both sensitized groups suggest that exposure to peanut protein through barrier-disrupted skin (ear and back) is sufficient to facilitate sensitization to peanut.

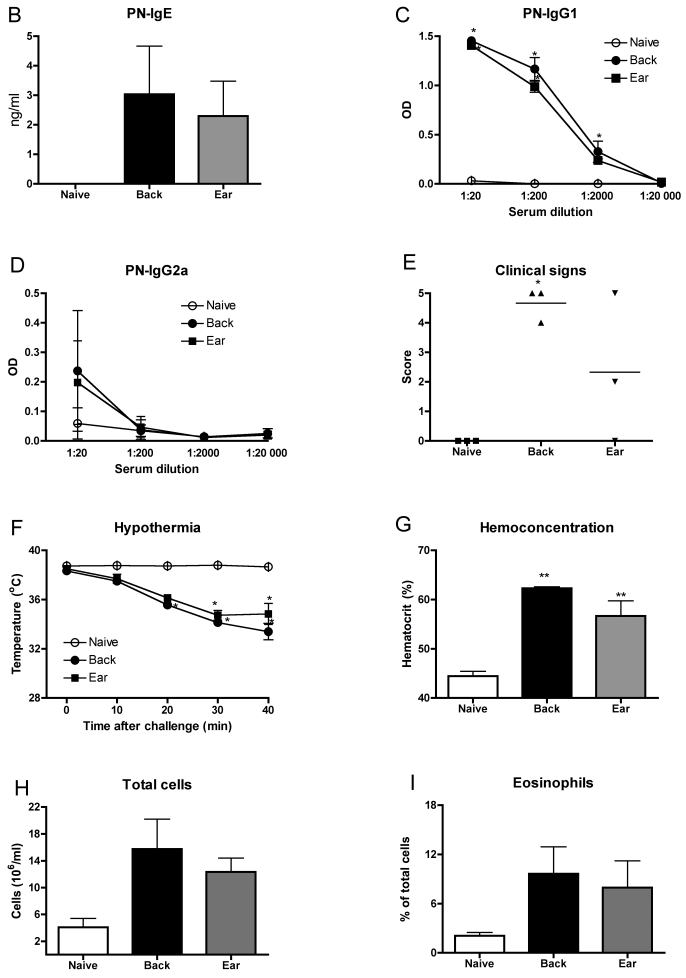

Figure 5 b-i: Epicutaneous Sensitization Through Exposure Via the Ear or Dorsal Skin. Mice were exposed to 200 µg peanut protein via the ear or back skin for 10 consecutive days. PN-specific IgE (b), IgG1 (c), and IgG2a (d) were measured in the serum two weeks following the final exposure. Sensitized mice were challenged i.p. 1 week following final exposure and clinical sings of anaphylaxis (e), core body temperature (f), and hematocrit 40 minutes after challenge (g) were measured. Total cell number (h) and percentage of eosinophils (i) in the peritoneal lavage were evaluated. 500 cells were counted under 40X magnification Data presented as m±SEM, n=3 mice per group. *p<0.05

DURATION OF EXPOSURE

Similar to traditional models of oral sensitization, epicutaneous sensitization protocols generally involve exposure to antigen over a period of several weeks, and this lengthy duration limits both the investigation of acute interventions and during the relatively brief infancy period in mice. Furthermore, significant changes in immune cell competency have been observed in the skin during the first 7-14 days of life [95]. Thus, the second objective in establishing an epicutaneous sensitization model was to determine the minimum amount of time required to elicit robust and consistent sensitization. To

determine whether acute exposure to CPE through tape-stripped skin was sufficient to induce sensitization, mice were exposed to either 7 or 10 continuous days of epicutaneous exposure through tape-stripped dorsal skin (Figure 6 a). These durations were chosen based on a previously developed model of oral sensitization, where both 7 and 10 days, but not less, of oral exposure to peanut and cholera toxin were capable of inducing robust sensitization in adult mice.

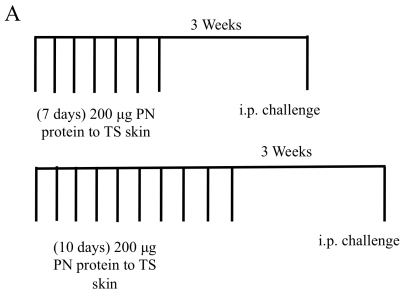

Figure 6 a: Schematic of Shortened Epicutaneous Sensitization Protocols. Mice were exposed to 200 µg of peanut protein via tape stripped skin for 7 or 10 consecutive days.

Three weeks following epicutaneous CPE exposure on tape-stripped skin, levels of peanut-specific IgG1, IgG2a, and IgE in the serum were evaluated by ELISA. The 10-day exposed group showed an increase in peanut-specific IgE, that, similar to 3 weeks of exposure, was not statistically significant (Figure 6 b). Conversely, levels of peanut specific IgG1, but not IgG2a were significantly increased (Figure 5 c,d). In contrast, 7 days of CPE exposure did not induce significant levels of IgG1, IgG2a, or IgE (Figure 6 b-d). Thus, only 10 consecutive days of epicutaneous exposure to CPE induced significant Th2 sensitization.

Following i.p. CPE challenge, mice sensitized for 10 days showed clinical signs ranging from reduced motion to motionless. The range of clinical scores for mice exposed for 10 days was 0-3, whereas clinical scores for mice exposed for 3 weeks was 4-5. As expected, mice that were exposed for 7 days showed no clinical signs of anaphylaxis (Figure 5 e). The 10 day exposed group had an average temperature decrease of 5^{o}C, compared to an average decrease of 7^{o}C in mice exposed to 3 one-week modules of sensitization (Figure 5 f). The 7 day exposed group did not develop a decrease in temperature following i.p. challenge (Figure 5 f). Furthermore, hematocrit levels assessed 40 minutes following challenge from mice that were sensitized for 10 days were significantly increased (54%), compared naïve (43%) (Figure 5 g). No increase in hematocrit was detected in 7 day exposed mice. No mice in the 10-day sensitized group reached endpoint, compared to 66% of the 3 week sensitized group (data not shown). Overall, 10 days of sensitization resulted in a more variable rate of sensitization (67%-80% responders in C57BL/6 mice) than mice exposed for 3 one-week modules (100% responders). However, the 10-day epicutaneous sensitization group showed consistent and robust allergic responses in all parameters measured (immunoglobulins, clinical score, temperature, hematocrit).

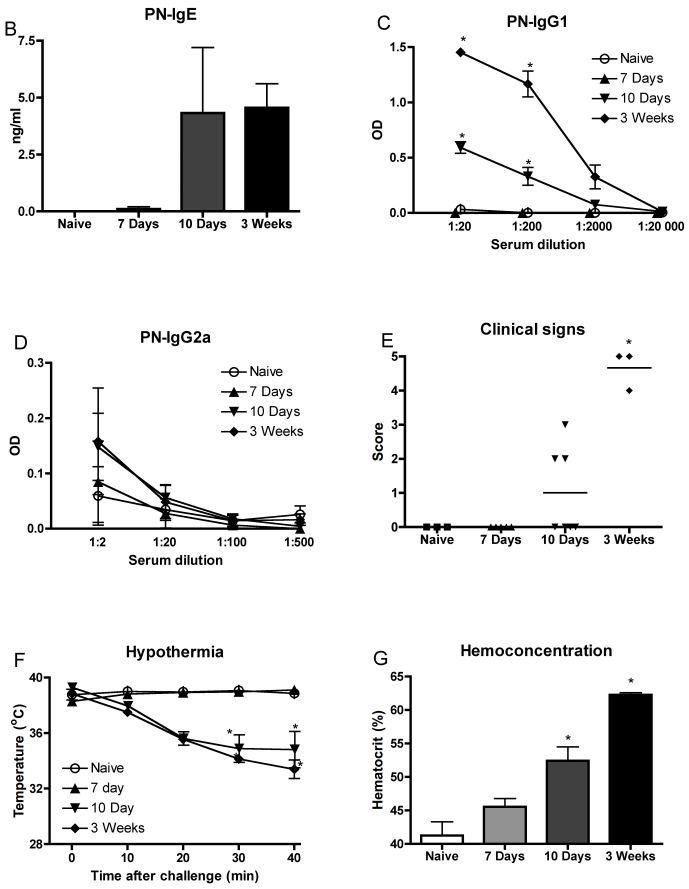

Figure 6 b-g: Duration of Epicutaneous Exposure Influences Sensitization. Levels of serum immunoglobulins following epicutaneous exposure to peanut through tape stripped skin for 7 or 10 consecutive days. Peanut-specific IgE (b), IgG1 (c), and IgG2a (d) were measured by ELISA. Anaphylaxis was evaluated following systemic challenge 3 weeks after epicutaneous exposure to peanut. Clinical signs of anaphylaxis (e), hypothermia (f), and hematocrit 40 minutes after challenge (g) were evaluated. Data presented as m±SEM, n=3-8 mice per group. *$p<0.05$

In conclusion, 10 days, but not 7 days of exposure to CPE through tape-stripped dorsal skin induces a robust, and consistent allergic response to peanut. Therefore, 10-days of epicutaneous sensitization was used for all following experiments.

INFLUENCE OF STRAIN-DEPENDENT TH2 SUSCEPTIBILITY

Next, the influence of genetic background was investigated in order to determine the consistency of the model in the strains of mice most commonly used in peanut allergy research. C57BL/6 (Th1-biased), BALB/c (Th2-biased), and C3H/HeJ (Th2-biased, TLR-4 deficient) mice were subjected to 10 days of epicutaneous peanut exposure through tape-stripped skin.

Three weeks following epicutaneous sensitization, serum levels of peanut-specific IgG1, IgG2a, and IgE were analyzed by ELISA. Levels of peanut-specific IgE were significantly increased in both C3H/HeJ mice and BALB/c, but not in C57BL/6 mice (Figure 7 a). Peanut-specific IgG1 titers were highest in the Th2 susceptible C3H/HeJ strain, and significantly increased similarly in both C57BL/6 and BALB/c mice (Figure 7 b). The Th1-type immunoglobulin, IgG2a was not significantly increased in any of the three strains (Figure 7 c). Thus, BALB/c and C3H/HeJ mouse strains show significantly higher serum levels of peanut-specific IgE than the Th1 prone C57BL/6 strain. However, Th2 susceptibility did not have a significant impact on the development peanut-specific IgG1.

40 minutes following i.p. challenge, clinical scores in C3H/HeJ mice ranged from 3 to 5, with approximately 20% of mice reaching endpoint (seizure or death). In contrast, BALB/c mice scored between 1 and 3, with most mice progressing to reduced motion, and with no mice reaching endpoint. C57BL/6 mice also scored between 1 and 3, with most mice scoring between 1 and 2 (Figure 7 d). C3H/HeJ mice showed a dramatic decrease in temperature (up to a 10 degrees Celsius), and both BALB/c and C57BL/6 mice also had significantly decreased temperatures 40 minutes following challenge (5 and 4 degree Celsius respectively) (Figure 7 e). Hemoconcentration was significantly increased in all sensitized groups, with C3H/HeJ and BALB/c mice having the highest hematocrit values (62% cell volume), and C57BL/6 mice increasing to 52% (Figure 7 f).

The late-phase response was evaluated 72 hours following challenge (Figure 7 g,h). Recruitment of eosinophils in C57BL/6 was significantly increased; however, while the total cell number was increased compared to the naïve group, it did not achieve statistical significance (Figure 7 h). BALB/c mice showed an increase in both eosinophils (2 fold increase) and total cell number (2.5 fold increase), however, due to large variability in this readout the results were not statistically significant. Interestingly, C3H/HeJ mice, which showed the strongest primary response, did not show increases in either total cell number or eosinophil recruitment at 72 hours (Figure 7 g,h). This may be explained by a strain dependent tendency to mount poor eosinophil responses, as C3H/HeJ mice have been shown in a model of allergic asthma to also mount less robust eosinophil responses [108]. Alternately, the kinetics of the late phase response may be different in C3H/HeJ mice compared to C57BL/6.

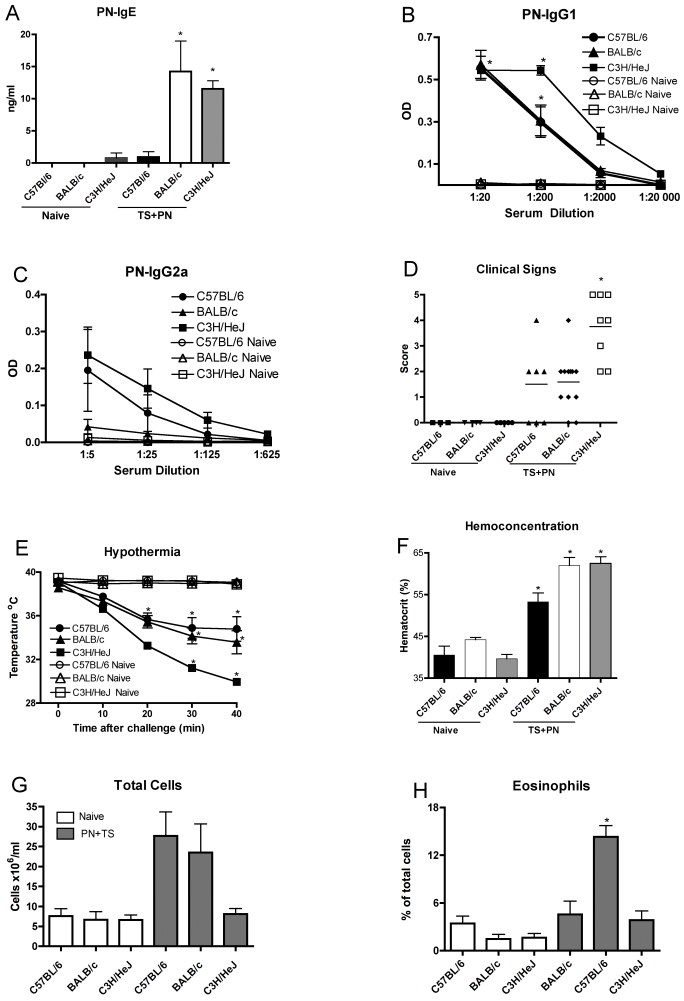

Figure 7: Impact of Strain on Epicutaneous Sensitization. Levels of serum immunoglobulins in C3H/HeJ, BALB/c, and C57BL/6 mice 3 weeks after epicutaneous sensitization. Peanut-specific IgE (a), IgG1 (b), and IgG2a (c) were measured by ELISA. Clinical signs of anaphylaxis (d), core body temperature (e), and hematocrit at 40 minutes (f), were evaluated following i.p. challenge. The late phase response was evaluated 72 hours after challenge. Total cell number (g) and percentage of eosinophils (h) were evaluated at 40X magnification. Data presented as m±SEM, n=3-8 mice per group. *p<0.05

In conclusion, C3H/HeJ mice had the most robust IgG1 sensitization and anaphylactic response of the three strains studied. IgE was also significantly increased in C3H/HeJ mice. BALB/c mice showed strong sensitization, with significant increases in both peanut-specific IgG1 and IgE, as well as a more robust anaphylactic response than C57BL/6 mice. The increase in peanut-specific IgE in BALB/c mice compared to C57BL/6 was associated with increased anaphylactic responses. Furthermore, both BALB/c and C3H/HeJ mice showed a 90-100% sensitization rate. Despite showing the greatest level of sensitization and anaphylactic responses, due to the unavailability of knockout strains on a C3H background, we chose to use this strain for specific

experiments, and only when a very robust response was required. Since BALB/c mice showed more consistent and robust anaphylactic responses than C57BL/6 mice, as well as significantly increased levels of peanut specific IgE, BALB/c mice were chosen to allow greater flexibility for future studies in neonatal mice.

INFLUENCE OF BARRIER INTEGRITY

Epicutaneous sensitization protocols disrupt barrier integrity either through tape stripping or application of an occlusive patch. Our next goal was to determine whether barrier disruption was required for epicutaneous sensitization during a 10-day exposure to peanut. Removal of the stratum corneum following tape stripping results in a slight increase in epidermal thickness, which is comparable to tape stripping in addition to CPE application (Figure 8 a). Using C3H/HeJ mice, which show the most robust sensitization and peanut-induced anaphylaxis, we responses between adult mice that were exposed to CPE for 10 consecutive days through tape stripped skin with mice that were exposed through normal, shaved skin. Three weeks following the final exposure, serum was collected for immunoglobulin analysis, and mice were challenged i.p. with CPE. In contrast to the mice that were exposed via tape stripped skin, mice exposed via normal skin did not develop either peanut-specific IgG1 or IgE (Figure 8 b,c). Furthermore, exposure via normal skin did not induce any signs of anaphylaxis, hypothermia, or increase in hematocrit following systemic CPE challenge (Figure 8 d-f). Thus, barrier disruption is necessary to induce sensitization to peanut through the skin.

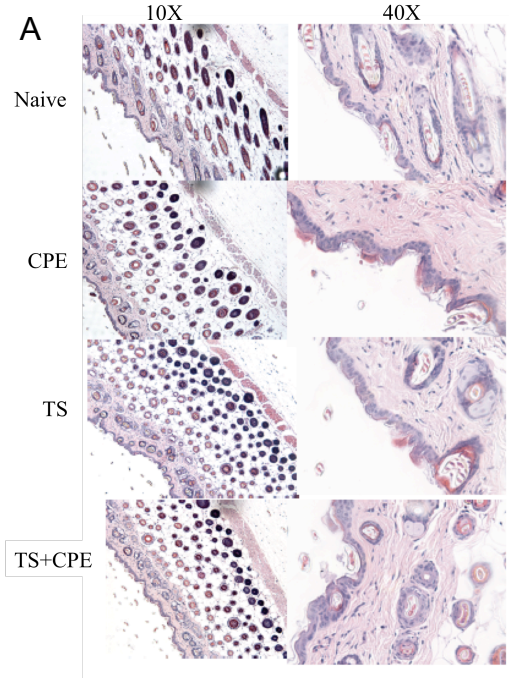

Figure 8 a: H&E stained skin sections from BALB/c mice. Skin from naïve, CPE treated, tape stripped, and tape stripped with CPE application were evaluated.

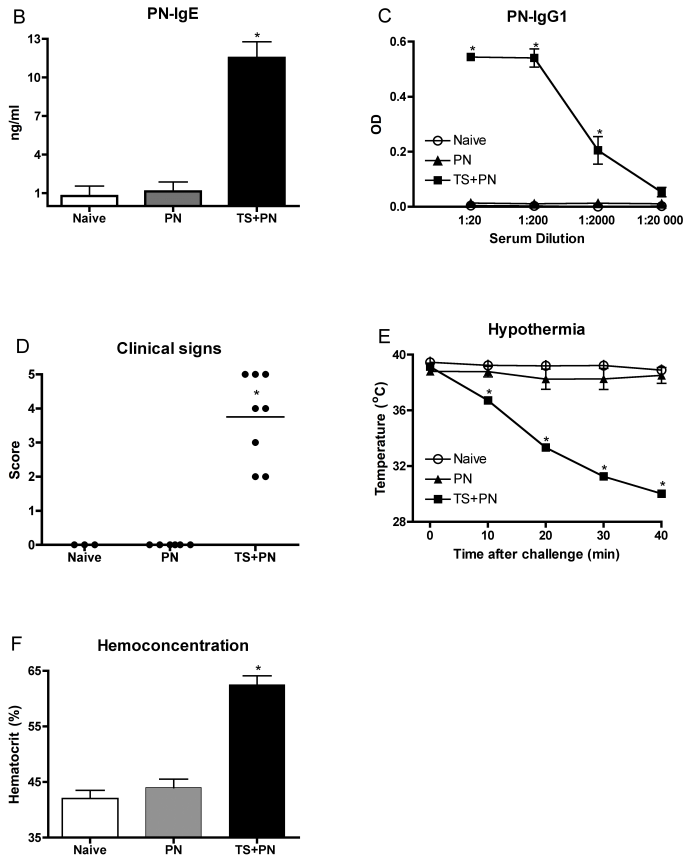

Figure 8 b-f: Impact of Barrier Integrity of Epicutaneous Sensitization. Levels of serum peanut-specific IgE (b) and IgG1 (c) 3 weeks after epicutaneous exposure to peanut through tape stripped or intact skin for 10 consecutive days were measured by ELISA. Following i.p, challenge, clinical signs of anaphylaxis (d), core body temperature (e), and hematocrit 40 minutes after challenge (f) were evaluated. Data presented as m±SEM, n=3-8 mice per group. *$p<0.05$

INFLUENCE OF EPICUTANEOUS SENSITIZATION ON SUSCEPTIBILITY TO ANAPHYLAXIS-ORAL CHALLENGE

To determine whether sensitization through the skin, unlike oral sensitization, facilitates anaphylaxis to oral peanut challenge, we used C3H/HeJ mice, which were shown to have the strongest anaphylactic responses following i.p. challenge. Mice were sensitized epicutaneously, and IgG1 was measured 3 weeks following the final CPE exposure (Appendix A-3). C3H/HeJ mice were orally challenged with 70 mg of CPE by gastric gavage in 500 µl PBS. Mice monitored for 40 minutes did not show clinical signs of anaphylaxis or hypothermia (Figure 9 a,b). Following the 40 minutes of monitoring, mice were challenged i.p. to confirm sensitization, and monitored again for 40 minutes. All i.p. challenged mice showed a significant decrease in body temperature (Appendix A-

4). Thus, epicutaneous sensitization in mice does not produce susceptibility to oral challenge to peanut, even in genetically susceptible mice.

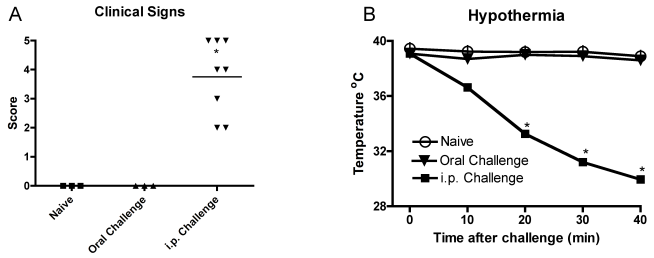

Figure 9: Oral Challenge Following Epicutaneous Sensitization. Mice were challenged by gavage with 50 mg peanut protein, followed by a second dose of 70 mg 20 minutes later, and monitored for clinical signs of anaphylaxis (a) and core body temperature (b). Data presented as m±SEM, n=3-8 mice per group. *p<0.05

IMPACT OF AGE AT FIRST EXPOSURE ON THE DEVELOPMENT OF PA

Following the establishment of an appropriate model of epicutaneous sensitization to peanut, we began investigating sensitization during early life. BALB/c mice were epicutaneously exposed to CPE for 10 consecutive days through tape stripped dorsal skin, beginning at one day after birth or at 2 weeks of age (Figure 10 a). Three weeks following CPE exposure, serum levels of peanut-specific immunoglobulins were analyzed by ELISA. Mice exposed beginning on day 1 of life did not develop peanut-specific Th2 immunoglobulins, IgE, IgG1, or the Th1 immunoglobulin, IgG2a (Figure 10 b,c). In contrast, mice exposed to CPE epicutaneously beginning at 2 weeks of age developed significant levels of peanut-specific IgG1 that tended to be similar to levels observed in adults (Figure 10 c). Peanut-specific IgE titres were similarly increased in mice sensitized at 2 weeks, or as adults (Figure 10 b). Levels of peanut-specific IgG2a were not significantly increased in mice epicutaneously exposed to CPE at any age, suggesting a primarily Th2-biased response (Figure 10 d).

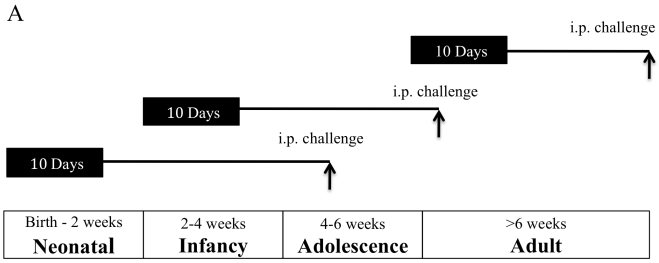

Figure 10 a: Schematic Showing Age at First Epicutaneous Exposure to Peanut. Mice were exposed to CPE through tape stripped skin for 10 consecutive days, beginning within the first 24 hours of life, at 2 weeks or age, or at 6-8 weeks of age.

Upon systemic (i.p.) CPE challenge, mice that were exposed to CPE beginning on day one of life showed no clinical signs of anaphylaxis (Figure 10 e). No hypothermia

was detected within 40 minutes of challenge (Figure 10 f), and hematocrit was not significantly different from the naïve group (Figure 10 g). In contrast, mice sensitized at 2 weeks of age showed clinical signs of anaphylaxis that were similar to mice sensitized as adults (Figure 10 e) and, similarly, showed a significant decrease in temperature following i.p. CPE challenge (4^{o}C decrease) (Figure 10 f). The increase in hematocrit was slightly less but not significantly different from adult levels (55% vs 65%) (Figure 10 g).

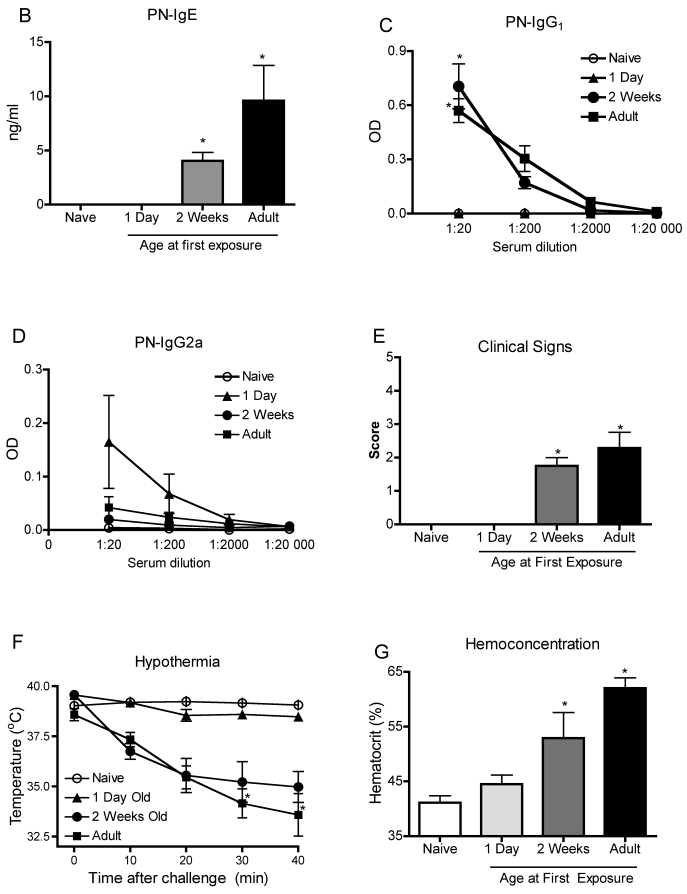

Figure 10 b-g: Impact of Age on Epicutaneous Sensitization. 3 weeks after epicutaneous peanut exposure starting at 1 day, 2 weeks, or 6-8 weeks of life, levels of peanut-specific IgE (b), IgG1 (c), and IgG2a (d), were evaluated by ELISA. Mice were monitored for clinical signs of anaphylaxis (e), core body temperature (f), and hematocrit was measured 40 minutes after challenge (g). Data presented as m±SEM, n=3-8 mice per group. *p<0.05

Thus, in terms of both peanut-specific immunoglobulins and clinical symptoms of peanut-induced anaphylaxis, very early epicutaneous exposure (during the first 10 days of life) does not induce sensitization, and exposure during infancy (after 2 weeks of life) results in a slightly diminished, but still clearly clinically relevant sensitization.

CHAPTER 5: DISCUSSION

The increasing prevalence of peanut allergy and lack of therapeutic options, with the exception of epinephrine administered after the onset of an anaphylactic reaction, uncovers a striking need to expand knowledge concerning the development of allergic sensitization. The processes involved in the elicitation of allergic sensitization remain largely unknown, especially during the highly relevant neonatal/ infancy period. The major focus of this thesis work was to develop models with the potential to be used in the investigation of the impact of both short-term interventions, such as an acute infection, antibiotic treatment, or NSAID use; and also to investigate sensitization early in life, a critical period when in most instances peanut allergy likely develops. Conventional animal models of peanut allergy require a period of weeks to more than a month to establish sensitization, thus precluding the investigation of short-term interventions. Therefore, the development of a model of oral sensitization that requires only 10 consecutive daily exposures to peanut has some obvious advantages. Furthermore, the development of a model of epicutaneous sensitization that also requires only 10 consecutive exposures provides the opportunity to study sensitization during the brief neonatal period in mice.

IMMUNOGLOBULINS AND ANAPHYLAXIS

Our newly developed models of sensitization to peanut generate peanut-specific immunoglobulins and result in the elicitation of anaphylactic responses upon re-exposure to peanut. In the oral model, peanut-specific IgE does not reach statistical significance. In the epicutaneous model, IgE levels were strain-dependent, with Th2 prone BALB/c and C3H/HeJ mice developing significantly greater immunoglobulin levels and a more robust anaphylactic response that C57BL/6 mice. As anaphylaxis is highly dependent on immunoglobulins, the lower level of peanut-specific IgE in our 10 day models is likely responsible for the less robust decrease in temperature during anaphylaxis, compared to conventional models of sensitization. In general, class switching to IgE requires more time than switching to IgG1, and *in vitro* studies have shown that a 3 fold increase in duration of exposure to IL-4 is required to induce maximal IgE responses compared to IgG1 [109]. The 10-day sensitization models may allow for less exposure of B cells to IL-4, thus limiting the IgE response. Alternatively, inflammation caused by daily administration of adjuvant in oral sensitization or more frequent tape stripping in the epicutaneous model may activate regulatory mechanisms to control immune responses. In any case, our findings suggest that an acute event, such as a GI infection or skin rash during infancy, has the potential to induce sensitization to peanut.

GENETICS

Genetic susceptibility to atopy, as well as peanut allergy, has been documented in many clinical studies. Indeed, peanut allergy shows a strong familial correlation, with monozygotic twins showing a 64.3% concordance rate, whereas dizygotic twins with

peanut allergy only occurred in 6.8% of those studied [110]. Consistent with these observations, our data show that genetic background significantly affects the degree of sensitization and, ultimately, the severity of PIA in C57BL/6, BALB/c, and C3H/HeJ mice epicutaneously sensitized to peanut**.** In general, C57BL/6 mice tend to have stronger Th1 responses and weaker Th2 responses than BALB/c mice [111, 112]. C3H/HeJ mice are prone to Th2 responses, and are frequently used in food allergy models [113].

We found that genetic susceptibility to Th2 responses is correlated with the level of peanut-specific IgE observed in the 10-day epicutaneous sensitization model. In this protocol, both BALB/c and C3H/HeJ mice develop, compared to C57BL/6, higher parameters of sensitization (significant IgE and higher IgG1). Previous studies from our laboratory have shown that sensitization to peanut is IL-4 dependent, as IL-4 deficient mice do not develop significant levels of peanut-specific immunoglobulins following oral sensitization (Llop et. al. manuscript submitted). Furthermore, BALB/c $CD4^+$ T cells have been shown to produce more IL-4 than C57BL/7, suggesting that IL-4 may contribute to the increased IgE in BALB/c mice. In addition, cultured splenocytes from peanut-sensitized C3H/HeJ mice have been shown to produce higher levels of IL-4 and lower levels of IFN-γ than BALB/c mice following *in vitro* restimulation [113]. Consistent with these findings, in our model, the C57BL/6 strain develops PIA that is clearly less severe than in both the BALB/c and C3H/HeJ strains. The differences observed in these various genetic strains of mice illustrate the diversity of immunological and clinical responses in a heterogeneous population.

INDOMETHACIN: PGE2 CONDITIONING OF DCS

NSAIDs inhibit the production of prostaglandins in the small intestine, resulting in decreased mucus production and increased intestinal permeability. It has been proposed that increased translocation of bacteria and pancreatic proteases lead to an inflammatory response. We hypothesized that NSAID treatment would facilitate sensitization to co-administered peanut. It has been shown that administration of indomethacin to gluten-sensitized mice significantly increases intestinal permeability, as well as histological parameters of intestinal damage [55]. However, our data show that following treatment with indomethacin at doses that have been shown to impair intestinal barrier function, but not induce robust inflammation, sensitization to peanut did not occur. Thus, increased translocation of protein does not appear to be sufficient to induce peanut sensitization. Indomethacin inhibits both COX-1 and COX-2, restricting the production of prostaglandins. Under normal conditions, constitutive activation of COX-2 in the small intestine ensures a consistently high level of PGE2, with DCs in the intestine producing nearly 100 times more PGE2 than DCs in the spleen. This is of significance because PGE2 has been shown to suppress IL-12 and Th1 responses (IFN-a by pDCs), but also to increase MHC II expression, migration of DCs, and IL-4 production by T cells [114], [115]. Therefore, it is possible that while NSAID treatment may increase intestinal permeability, it also reduces susceptibility to Th2 responses by impairing DC programming and preventing IL-4 induced Th2 differentiation by inhibiting PGE2

production. Our data suggest that it is likely that additional inflammatory signals, or a specific type of inflammation are required to induce sensitization to food antigens via the digestive tract.

OTHER POTENTIAL ROLES OF NSAIDS (ORAL TOLERANCE)

Rather than actively inducing sensitization to foods, NSAIDs may inhibit oral tolerance and increase future susceptibility to sensitization. A number of studies have shown that NSAID treatment is capable of preventing oral tolerance in murine models, including the prevention of oral tolerance to ovalbumin [116]. In this study, mice overexpressing the T cell receptors for OVA display intestinal inflammation and increased T cell proliferation following feeding with ovalbumin only when coadministered with indomethacin or the selective COX-2 inhibitor, NS-398. It should be noted that, in this situation, a considerable number of antigen specific T cells were in place before barrier disruption with NSAIDs. It is unknown whether these mice also developed allergic sensitization to OVA. This is of particular relevance as PGE2 has been shown to both induce FOXP3 expression in $CD4^+$ Tregs, as well as mediating inducible Treg suppression of $CD4^+$ effector T cells [117]. Furthermore, COX-2 is involved in the induction of FoxP3+ Treg differentiation in the intestine [62]. Once established, oral tolerance provides antigen specific Tregs that may aid in the prevention of inappropriate or allergic responses to that food. The inability of indomethacin to induce sensitization to peanut in our model may suggest that while NSAIDs may have the capacity to prevent oral tolerance, they are not sufficient to induce sensitization. Furthermore, it is possible that in addition to altering the integrity of the intestinal mucosa, the presence of an excess of antigen-specific T cells is necessary for NSAID induced responses to food antigens. Thus, NSAIDs may play an indirect role in allergic sensitization through preventing the development of antigen-specific T regulatory cells and oral tolerance. Future work should clarify this role, as it may be prudent to avoid introduction of new foods to children during NSAID treatment before oral tolerance has been established.

ORAL CHALLENGE

Ovalbumin and beta-lactoglobulin are capable of inducing anaphylactic reactions following oral challenge in sensitized mice. However, the elicitation of anaphylaxis to peanut relies on systemic (i.p or i.v.) challenge [15, 118]. A small number of studies, all from the same group, have successfully achieved responses to oral challenge with peanut; however, the readouts used were less robust than with systemic challenges, and have not been readily replicated by other groups including ours. In attempting to achieve anaphylaxis following oral challenge, groups have used bicarbonate to decrease protein digestion and high amounts of peanut protein (comparable to 18 peanuts) in C3H/HeJ mice, which are highly susceptible to peanut allergy [119]. Achieving a model in which oral challenge induces anaphylaxis would be beneficial because it would presumably more closely represent human peanut allergy. Furthermore, the route of challenge may activate different cellular pathways of anaphylaxis. Therefore, we evaluated whether

treatment with indomethacin would increase susceptibility to oral challenge in peanut-sensitized mice. In addition, we investigated whether sensitization through the skin would increase susceptibility to anaphylaxis following oral challenge.

We postulated that treatment with high doses of NSAIDs *in vivo* would decrease the threshold dose of peanut required to elicit of anaphylaxis in peanut-sensitized mice exposed orally to peanut protein. It has been shown that overexpression of IL-9, which leads to increased intestinal permeability, enhances diarrheal responses, increases vascular permeability and increases hematocrit following oral challenge in ovalbumin sensitized mice, compared to sensitized wild type mice [51]. However, even with the use of exceptionally high doses of both indomethacin and peanut protein, we did not detect any signs of anaphylaxis.

Increases in intestinal permeability may not be required or sufficient to facilitate anaphylaxis in sensitized mice. Indeed, oral gavage with peanut and cholera toxin, which is known to significantly increase intestinal permeability and levels of orally administered antigen in serum, does not induce anaphylaxis following oral peanut administration in peanut-sensitized mice (Magnusson et al. 1985). This study, in addition to our own observations, suggests that the administration of protein with agents that increase intestinal permeability (indomethacin or cholera toxin) is not sufficient to induce anaphylaxis in peanut-sensitized mice.

EPICUTANEOUS SENSITIZATION

Following recent clinical evidence linking peanut allergy to epicutaneous peanut exposure during infancy, we developed a model of epicutaneous sensitization that could be applicable in the neonatal setting. Our newly developed model that induces sensitization to peanut through the skin with only 10 consecutive days of exposure, supports the notion that the skin may be a relevant route of sensitization in children [11, 12]. Previously established models of epicutaneous sensitization to peanut assessed peanut-specific immunoglobulins as well as delayed type hypersensitivity responses, however, they did not assess anaphylactic reactions, a hallmark of peanut allergy following systemic challenge [87]. Models of epicutaneously-induced food allergy to other food proteins (ovalbumin, hazlenut) lead to the development of anaphylactic symptoms, but utilize lengthy sensitization protocols (up to 42 days) that alternate weeks of exposure with weeks of rest [90, 120]. Clearly, these protocols are inadequate to examine epicutaneous sensitization in neonates.

In light of the recent evidence linking cutaneous exposure to peanut during acute disruptions of the epidermis during infancy, investigation in an animal model is especially merited. The applicability to studies during development is one of the greatest practical strengths of the 10-day epicutaneous sensitization model.

NEONATAL HYPORESPONSIVENESS

The finding that neonatal mice are hyporesponsive to peanut sensitization using our epicutaneous model was particularly surprising, as literature suggests a strong Th2

predisposition early in life. Robust sensitization was observed in mice when exposure began as early as 2 weeks of age. Interestingly, these findings parallel the development of the epidermal APC compartment. Langerhans cells have been shown to develop a mature phenotype following tape stripping, and to migrate to the draining lymph node only when tape stripping was combined with the application of antigen in adult mice [70]. This suggests that LCs may be involved in the initiation of an immune response following epicutaneous immunization.

In the mouse, LCs appear in fetal skin at day 18 of gestation, and are present at high levels (based on MHC II expression) immediately following birth. However, the LC network is not fully matured until 14 days following birth [95]. At day 3 of life, LCs remain as globular shaped cells, expressing MHC II primarily on the cell body [94, 95]. At day 7, dendritic processes begin to form, and MHC II expression on processes increases. However, it is not until day 14 that the network reaches adult status. In addition, the expression of DEC207, a marker of antigen scavenging ability is absent at day 3 of life, and increases progressively until day 14. It has been shown in a number of murine models that these developmental events correlate with the ability to transport antigen to the draining lymph nodes, as well as the initiation of immune responses [94, 95, 121]. For example, 3 and 7 day old mice exposed to contact sensitizers show a decreased ability to transport antigen (FITC) to the lymph node, and a reduced susceptibility to epicutaneous sensitization as adults with antigens that they were exposed to as neonates, suggesting that epicutaneous exposure early in life may have generated a tolerogenic, rather than inflammatory response [95]. We also find that mice exposed to peanut through the skin for the first 10 days of life do not become sensitized. Thus, the first 2 weeks of life represent a critical period in the development of the capacity to mount adult-like immune responses to cutaneously delivered antigen. This may, in part, explain the rapid acquisition of susceptibility to epicutaneous peanut sensitization in 2 week old compared to newborn mice. It is unknown whether epicutaneous exposure to peanut during the first 10 days of life is capable of inducing tolerance, but is of significant interest for future studies.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

ORAL SENSITIZATION AND INDOMETHACIN

The first goal of our study was to develop a short model of oral sensitization, using cholera toxin as an adjuvant. We determined that oral exposure to peanut in the presence of cholera toxin for 10 consecutive days could induce robust sensitization, and anaphylaxis following systemic challenge. Thus, this model is useful to investigate short-term interventions (infection, drug treatment such as NSAIDs or antibiotics, etc) on the development of sensitization or tolerance to peanut.

We also demonstrated that acute use of an NSAID (indomethacin), at doses previously shown to increase intestinal permeability, does not result in allergic sensitization to peanut. Thus, indomethacin treatment is unlikely to contribute significantly to allergic sensitization.

Lastly, treatment with a dose of indomethacin that is capable of increasing intestinal permeability did not increase susceptibility to anaphylaxis following oral challenge in peanut-sensitized mice. This suggests that mechanisms other than antigen passage across the intestinal epithelium are critical in the elicitation of anaphylaxis following oral exposure. An experiment addressing the change in specific permeability to peanut, using an Ussing chamber, would further clarify the role of intestinal permeability in facilitating anaphylaxis following oral exposure.

EPICUTANEOUS SENSITIZATION

Based on evidence linking cutaneous exposure to peanut with peanut allergy in children, we developed a short model of epicutaneous sensitization that would facilitate the investigation of sensitization to peanut throughout the developmental period. Following 10 consecutive days of peanut exposure through tape stripped skin, adult mice developed significant levels of peanut-specific immunoglobulins, as well as a robust anaphylactic response following systemic challenge. These effects were most pronounced in BALB/c and C3H/HeJ mice, which are predisposed towards a Th2 response. To our knowledge, this is the first system of epicutaneous sensitization to peanut that induces anaphylaxis following systemic challenge, using an acute period of sensitization. These findings support the notion that the skin may be a relevant route of sensitization to peanut, especially in genetically predisposed individuals. Therefore, an important future direction is to determine the mechanisms that contribute to sensitization through this route.

An interesting question to investigate is whether LCs are required following for sensitization in our model. LCs are the only professional antigen presenting cell type in the epidermis; and consequently, have been the focus of a number of studies concerning immune responses in the skin. Recently, the role of LCs versus the dermal-resident DC subsets has been investigated in a number of models, with controversial results. Indeed, LCs have been found to be both dispensable and necessary for either the induction of tolerance or immunity in different experimental models [122]. This conflicting evidence is likely due to both a functional plasticity of LCs, as well as the fact that until recently, CD207 (langerin) was thought to be exclusively expressed on LCs, when it is now known to be found on a subset of dermal DCs as well.

It has been found that following application of contact sensitizers, LCs migrate to the draining lymph nodes, and generally peak in number in the LN approximately 4 days following stimulation. Thus, it is entirely plausible that LCs are essential for antigen delivery and/or T cell activation in our model of epicutaneous sensitization.

In addition to LC activation and migration playing a role in a number of epicutaneous protocols, we have generated some data that suggests that TGF-β, which regulates LC homeostasis, may be involved in epicutaneous sensitization to peanut. LCs are dependent on the autocrine production of TGF-β1 for both their development and survival in the epidermis. Indeed, TGF-β1 deficient mice do not develop LCs. [123-125]. In a preliminary study, we found that treatment with a pan-TGF-β neutralizing antibody during epicutaneous sensitization significantly reduced production of peanut-specific immunoglobulins, as well as anaphylactic responses. It is then possible that such

diminished responses were due to disruption of the LC network by TGF-β neutralization. Immunohistochemistry of the epidermis would be helpful to determine whether the LC network was compromised by this treatment, and flow cytometry of the draining LNs would permit the investigation of whether these LCs were able to migrate following tape stripping and peanut application. Furthermore, the use of a langerin-DTR mouse strain would be especially clarifying. These mice may be depleted of LCs for up to 7 weeks following treatment with diptheria toxin, and therefore permit the investigation of the role of LCs.

Following tape stripping and antigen application, keratinocytes rapidly produce an array of cytokines and other signals that may initiate the migration of LCs following application of peanut, or alternatively induce maturation of these cells. It has previously been shown that disruption of the skin barrier through different treatments induces a similar profile of pro-inflammatory cytokines, including TNF-α, IL-1α, IL-1β, and IL-6, as well as the TNF(p55) [92]. These cytokines provide an important point of reference, as they have been shown to increase following tape stripping, as well as via a more natural barrier disruption, such as that caused by a dietary deficiency in fatty acids. Consequently, an analysis of these and other cytokines in the epidermis via PCR in our model would yield relevant information concerning the role of potential immunostimulatory factors in epicutaneous sensitization.

TNF-α is produced by both keratinocytes and LCs in the epidermis, and intradermal administration of recombinant TNF-α has been shown to induce a significant increase in DC accumulation in the draining LNs, suggesting that it may act as a migration signal for LCs [126-128]. However, the potential impact of TNF-α is complicated by the increase in TNF in neonatal versus adult epidermal cell cultures, as neonates are non-responsive to peanut sensitization in our model [129]. Thus, inhibition of TNF-α, either through antibody depletion, or using a knockout or receptor knockout mouse, would be a reasonable approach to determine the potential role of TNF-α induced migration of LCs in epicutaneous sensitization to peanut.

Furthermore, IL-1 has also been shown to be involved in initiating LC migration to the LN, and is produced by both keratinocytes (primarily IL-1α) and LCs (primarily IL-1β) [126, 130]. Following treatment with either TNF-α or IL-1, LCs lose adhesion molecules and gain the ability to pass through the basement membrane. Thus, inhibition of IL-1, either through neutralizing antibody or using a knockout strain of mouse would determine whether IL-1-mediated migration of LCs is involved in epicutaneous sensitization. Based on the potentially synergistic roles of IL-1 and TNF-α in LC migration [131], dual depletion of both cytokines via antibody administration would also be necessary.

Additionally, GM-CSF, which is produced by keratinocytes but not LCs, is essential for maintaining LC viability in culture, and is also proposed to contribute to the activation of LCs [132]. Indeed, following culture with GM-CSF, LCs were able to induce significantly greater proliferation of T cell cultures [133]. Furthermore, it has been shown that GM-CSF is necessary for LC survival, and works synergistically with IL-1 to increase LC stimulatory capacity in mixed lymphocyte cultures [134].

NEONATAL EPICUTANEOUS SENSITIZATION

We showed, for the first time, that epicutaneous exposure to peanut for the first 10 days of life, in contrast to exposure at later times (2 weeks and adult), does not result in allergic sensitization. Indeed, mice exposed to peanut during the neonatal period did not develop peanut-specific immunoglobulins, or show any signs of anaphylaxis following systemic challenge. However, 10 consecutive days of peanut exposure beginning as early as 2 weeks of life results in significant levels of peanut-specific IgE and IgG1, and clinical signs of anaphylaxis that are nearly as robust as in the adult model. This phenomenon may be explained, in part, by the developmental kinetics of the cutaneous antigen presenting cells. Therefore, it is likely that the immaturity of the LC network also influences the ability of neonatal mice to become sensitized to peanut through the skin. Alternatively, it is possible that signals from keratinocytes, which influence the maturation of LCs are lacking immediately following birth, and are acquired early in life through interactions with the environment (microbial colonization, etc.).

Cytokines such as GM-CSF, IL-1, TSLP, IL-6 and TNF-α may drive maturation of skin resident APCs and initiation of sensitization to peanut. These cytokines have been shown to increase rapidly following barrier disruption of the *stratum corneum* in adult mice. The expression of most pro-inflammatory cytokines in neonatal skin following tape stripping or application of an allergen is unknown. Therefore, to determine which cytokines may be under-expressed, rt-PCR of the epidermis to determine mRNA levels of cytokines known or suspected to be involved in maturation of LCs or DCs would be most informative.

Administration of recombinant cytokines to neonates during epicutaneous exposure could be useful in determining whether a lack of immunostimulatory signals is responsible for the lack of sensitization during the neonatal period. One limitation of such an approach is that the volume that may be administered to a newborn limits the dose of cytokine that can be administered. An alternate approach would be to create adenoviral vectors containing the cytokine gene of interest, which can be applied to tape stripped skin to infect keratinocytes [135].

Furthermore, TGF-β may also play a role in neonatal hyporesponsiveness to epicutaneous sensitization. Expression of TGF-β mRNA in the skin has been shown to be very low in newborn rabbits, and increases significantly by adulthood. Therefore, it is possible that low levels of TGF-β in the skin during early infancy prevent the coordinated activation and maturation of Langerhans cells. This hypothesis may also be investigated with the use of recombinant cytokine or an adenovirus vector. Determining whether a lack of LC activating signals is responsible for the hyporesponsiveness observed in neonatal epicutaneous sensitization will provide strategies for controlling the cutaneous response early in life.

An alternative explanation for the inability to sensitize neonatal mice to peanut through the skin is that there may be active suppression of the APC compartment during infancy. In other words, that the LC network is actively prevented from maturing to restrict the induction of detrimental immune responses during the transition from a nearly sterile intra-uterine life, to the antigen- and microbial-rich environment outside the womb.

This hypothesis is supported by a report from Chang-Rodriguez et. al., showing that autocrine IL-10 produced by LC precursors actively inhibits maturation into LCs. Specifically, this study found that epidermal cell supernatatants taken from newborn mice inhibited the upregulation of MHC II and costimulatory molecule CD86 by 41-47%. Through antibody treatment and immunofluorescence analysis, it was ultimately discovered that IL-10 contributed significantly to this suppressive function, and that neonatal mice, but not adults show high levels of IL-10, primarily in $CD45^+$ cells in the epidermis, which were determined to be LCs or LC precursors [121]. Thus, the role of suppressive cytokines, such as IL-10 should be investigated in our neonatal epicutaneous sensitization model, either through an anti-IL-10 receptor antibody or IL-10 deficient mice. Difficulties in determining the efficacy of an anti-IL-10 or anti-IL-10R antibody, in addition to the aforementioned technical difficulties in antibody administration to newborn mice make an IL-10 knockout preferable to other protocols interfering with IL-10 signaling in our model.

Ultimately, we hope that these future investigations will provide an experimental framework to understand the induction of immune responses during the neonatal period. Currently, the skin is the focus of intense investigation as both a site for inducing immunity (both in allergy and vaccination), as well as the induction of tolerance. However, little is known concerning these responses during infancy. Investigation of these mechanisms may provide direction for the development of protocols to actively induce tolerance to protein through the skin. This could potentially be used prophylactically in at risk populations.

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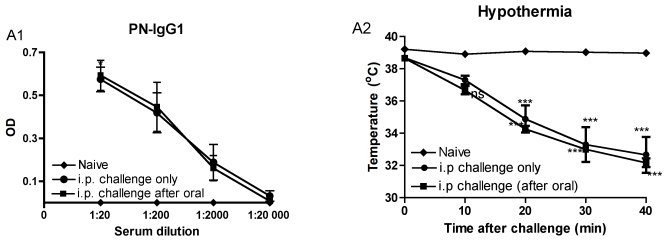
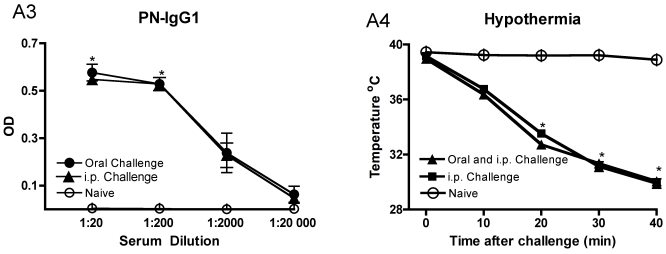
APPENDIX



A1: Comparable levels of peanut-specific IgG1 in sensitized mice challenged i.p. and orally
A2: Temperature following i.p. challenge in peanut-sensitized mice that were previously challenged orally with peanut.



A3: levels of peanut-specific IgG1 in epicutaneously sensitized mice before oral challenge. PN-IgG1 was measured by ELISA. Data presented as m±SEM. n=3-8 mice per group. *p<0.05

A4: core body temperature following i.p. challenge in epicutaneously sensitized, orally challenged mice. Mice were challenged i.p. 40 minutes after the second oral dose of peanut if no responses were observed. Data presented as m±SEM. n=3-8 mice per group. *p<0.05